

Antiallergic and Anti-Inflammatory Effects of a Novel I κ B Kinase β Inhibitor, IMD-0354, in a Mouse Model of Allergic Inflammation

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Key Words

Nuclear factor- κ B • IMD-0354 • I κ B kinase β inhibitor • Allergic inflammation • Airway hyperresponsiveness • Th1/Th2 balance • Immunoglobulin E • Bronchial asthma

Abstract

Background: Nuclear factor (NF)- κ B is a transcription factor known to regulate allergy-associated cytokine and chemokine production related to the induction of inflammation. I κ B kinase β (IKK β), which is responsible for activation of the NF- κ B pathway, may be an ideal molecular target to inhibit this process. IMD-0354 [N-(3,5-bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide] is an attractive novel IKK β inhibitor that prevents the production of inflammatory cytokines in various diseases, although it is not known if IMD-0354 is effective against allergic inflammation. This study aimed to elucidate the antiallergic effects of a newly synthesized IKK β inhibitor, IMD-0354, in a mouse model of allergic inflammation. **Methods:** We generated ovalbumin (OVA)-sensitized mice which were then challenged with OVA. IMD-0354 was administered intraperitoneally to therapeutic groups. Lung histopathology and the concentrations of cytokines and chemokines in bronchoalveolar lavage fluid (BALF) and supernatants of lung homogenates were determined. **Results:** Administration of IMD-0354 ameliorated

airway hyperresponsiveness and reduced the numbers of bronchial eosinophils and mucus-producing cells in OVA-sensitized mice. The total numbers of cells and eosinophils in BALF were also reduced by treatment with IMD-0354. Treatment with IMD-0354 inhibited the production of Th2 cytokines such as interleukin (IL)-5 and IL-13 and eotaxin in the airways and/or lungs of OVA-sensitized mice, but it did not affect the restoration of Th1 cytokines such as IL-12 and interferon- γ under the same experimental conditions. IgE production was also inhibited by IMD-0354. **Conclusion:** A specific IKK β inhibitor, IMD-0354, improved allergic airway inflammation and hyperresponsiveness in mice. IMD-0354 may have therapeutic potential for bronchial asthma.

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Introduction

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by infiltration of the lungs by eosinophils, lymphocytes, macrophages and other inflammatory cells. Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13 and chemokines such as eotaxin and IgE are known to be involved in the pathogenesis of bronchial asthma, while the production of Th1 cytokines is relatively suppressed [1].

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Recently, much attention has been paid to the role of the transcription factor nuclear factor (NF)- κ B, which is responsible for the induction of cytokine and chemokine gene expression and production. Activation of NF- κ B has been reported in the airways of patients with asthma [2, 3] and in animal models of asthma [4, 5]. Although the development of drugs targeting NF- κ B is expected to be useful for allergic inflammatory disease, there are currently no drugs available that specifically inhibit NF- κ B.

NF- κ B is normally found in the cytoplasm, held in an inactive state by its inhibitor chaperone I κ B α . Phosphorylation of I κ B α results in its ubiquitination and proteolysis, which then releases NF- κ B to promote gene transcription. The multisubunit I κ B kinase (IKK), responsible for phosphorylation of I κ B α , contains 2 catalytic subunits: IKK α and IKK β [6]. Since IKK β is an important kinase for controlling inflammatory responses, its inhibition may be an attractive strategy for developing new molecular-targeted drugs rather than direct inhibition of NF- κ B. In a previous report of IKK β inhibition in a mouse model of asthma, Birrel et al. [7] demonstrated that 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide (TPCA-1), a reagent that inhibits IKK β , blocked Th2 cytokine production in human airway smooth muscle in vitro and caused an antiasthmatic effect in a rat model of asthma. Ziegelbauer et al. [8] also demonstrated that a low-molecular-weight inhibitor of IKK β prevented allergic pulmonary inflammation and a Th2 response. However, these IKK β inhibitors have not yet been clinically developed.

IMD-0354 [N-(3,5-bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide] is a newly synthesized low-molecular-weight compound. It selectively inhibits IKK β in the IKK complex, which is only induced in inflammatory conditions. Onai et al. [9] reported that IMD-0354 attenuated myocardial ischemia/reperfusion injury without general or cellular toxicity in rats. IMD-0354 also ameliorated insulin resistance and upregulated plasma levels of adiponectin in KKAY mice [10]. Recently, we reported that IMD-0354 treatment inhibited bleomycin-induced pulmonary fibrosis in mice [11]. In a phase I study and preliminary proof of concept study, transcutaneous administration of an ointment containing IMD-0354 and IMD-1041, a prodrug of IMD-0354 that enables oral administration, was shown to be safe in patients with atopic dermatitis, hay fever and type 2 diabetes. Thus, IMD-0354 could be therapeutically useful for various inflammatory diseases through specific inhibition of IKK β . However, whether IMD-0354 is effective against airway allergic inflammation is not known.

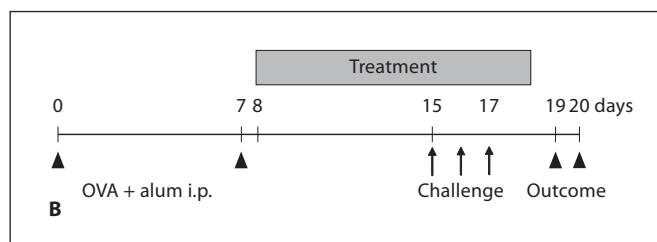
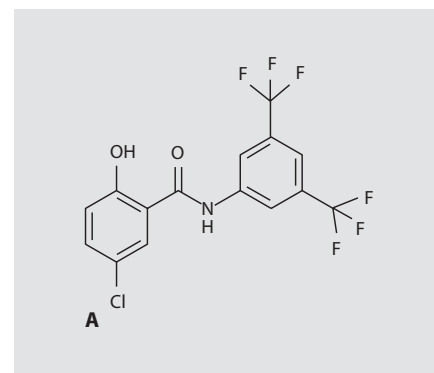


Fig. 1. **A** Structure of IMD-0354 (provided by the Institute of Medicinal Molecular Design Inc.). **B** Protocols for the mouse experiments.

In this study, we examined whether a specific IKK β inhibitor, IMD-0354, affected allergic airway inflammation, airway hyperresponsiveness (AHR) and production of IgE and Th1/Th2 cytokines in a mouse model of allergic inflammation. We report that IMD-0354 exerts antiallergic and anti-inflammatory effects by changing the profile of cytokine production via inhibition of NF- κ B activation.

Materials and Methods

Preparation of IMD-0354

A synthetic IKK β inhibitor, IMD-0354, was kindly provided by the Institute of Medicinal Molecular Design Inc. (Tokyo, Japan). IMD-0354 is a low-molecular-weight compound (molecular weight 383.7 Da; fig. 1A) [9, 11]. The specificity of IMD-0354 and its inhibitory activity against IKK β have been previously reported [9, 12]. For the present study, IMD-0354 powder (5 and 20 mg/kg) was dissolved in 0.5% carboxymethylcellulose (CMC; Sigma, Tokyo, Japan).

Mouse 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 our university [13]. The protocols for the mouse experiments are shown in figure 1B. Mice (2–6 for each experiment) were sensitized on days 0 and 7 by

intraperitoneal injection of 50 µg of OVA (grade V; Sigma-Aldrich, Tokyo, Japan) dissolved in 500 µl of saline and mixed with 1 mg of alum (Pierce, Rockford, Ill., USA). On days 15, 16 and 17, OVA-sensitized/challenged mice (OVA/-/OVA mice), OVA-sensitized/challenged mice with CMC treatment as a vehicle (OVA/CMC/OVA mice) and OVA-sensitized/challenged mice with IMD-0354 treatment (OVA/IMD/OVA mice) were challenged by exposure to an aerosol of 1% (w/v) OVA in saline for 20 min. Inhalation was conducted in a plastic chamber connected to the aerosol output from a Nissho nebulizer set (Alfresa Pharma Corporation, Osaka, Japan). Control mice were sensitized with OVA/alum and exposed to saline (OVA/-/Saline mice). In OVA/IMD/OVA mice, 5 or 20 mg/kg IMD-0354 (OVA/IMD 5/OVA and OVA/IMD 20/OVA mice, respectively) was administered intraperitoneally from day 8 to day 18 to achieve the maximum effect of IMD-0354, using a previous report [11] and preliminary examination (data not shown) as reference. As a vehicle injection model (OVA/CMC/Saline and OVA/CMC/OVA mice), 0.5% CMC without IMD-0354 was administered intraperitoneally during the same period.

Measurement of Airway Resistance

Forty-eight hours after the final challenge (day 19), airway resistance to β -methacholine (Mch) was assessed in conscious, unrestrained mice using unrestrained barometric whole-body plethysmography (Buxco Electronics, Troy, N.Y., USA) [14]. Enhanced pause (Penh) was evaluated as an index of airway resistance. Mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized Mch at different concentrations (6.3, 12.5, 25 and 37.5 mg/ml) was nebulized for 3 min, and readings were taken and averaged for 3 min after each nebulization.

Lung resistance (R_L) was also measured by restrained whole-body plethysmography (Buxco Electronics). On day 19, mice were anesthetized by 6 mg/ml 2,2,2-tribromoethanol (Sigma) with 2-methyl-2-butanol (Sigma). After mice were tracheostomized and cannulated with a 19-gauge tracheostomy tube, they were placed in the chamber for plethysmography and mechanically ventilated. Aerosolized Mch was administered for 10 s by an in-line aerosol delivery system at different concentrations (6.25, 12.5, 25 and 37.5 mg/ml). After each Mch challenge, data were continuously collected and values of R_L were taken to express the changes in these functional parameters. The data were expressed as the percentage of baseline in OVA/-/Saline mice.

Bronchoalveolar Lavage

On day 20, the mice were deeply anesthetized with 6 mg of 2,2,2-tribromoethanol and then sacrificed by cutting the axillary artery to obtain serum samples. Following sacrifice, bronchoalveolar lavage was performed twice with saline (0.8 ml) using a soft cannula. After counting the cell numbers in the bronchoalveolar lavage fluid (BALF) using the automated cell viability analyzer Vi-Cell (Beckman Coulter, Calif., USA), cells were cytospun onto glass slides and stained with Diff-Quick (Baxter, Miami, Fla., USA) for classification. The BALF supernatants were collected and stored at -80°C .

Histopathology

Following sacrifice, lung tissue was harvested from the thorax. Lung tissue was fixed in 10% formalin and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin

and eosin (HE) and periodic acid-Schiff (PAS). For assessment of infiltrating eosinophils in the subepithelium, the number of eosinophils was counted in a section stained with a modified Luna stain. The data were adjusted to the length of the basement membrane of the target bronchus, which was measured using an Olympus BX61 microscope (Tokyo, Japan) with Scion Image software. For the analysis of mucus-producing goblet cells, the PAS-positive area and the total epithelial area were measured using the Olympus BX61 with Scion Image software. The data were expressed as the percentage of the total area of epithelial cells stained with PAS.

Homogenization of Whole Lungs

Following sacrifice, the right lungs were frozen. Frozen tissue samples were solubilized by homogenization in lysis buffer (Cell Signaling Technology Inc., Danvers, Mass., USA) containing 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) using an Ultramicro homogenizer set (I.S.O. Inc., Yokohama, Japan). Samples of the homogenate were centrifuged at 15,000 rpm for 10 min. The supernatants were stored at -80°C .

Measurements of Total Protein and Cytokine Concentrations

Protein concentrations were determined by the Bradford method (Bradford Protein Assay, Sigma-Aldrich) using bovine serum albumin standards. IL-1 β , -4, -5, -12 and -13, interferon (IFN)- γ , tumor necrosis factor (TNF)- α and eotaxin were measured with commercial ELISA kits (R&D Systems, Minneapolis, Minn., USA). OVA-specific IgE was measured using the Dainippon Sumitomo mouse IgE ELISA (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) according to the manufacturer's instructions. The limits of sensitivity of the ELISA kits were as follows: IL-1 β , 3 pg/ml; IL-4, 2 pg/ml; IL-5, 7 pg/ml; IL-12 (p70), 4 pg/ml; IL-13, 1.5 pg/ml; IFN- γ , 2 pg/ml; TNF- α , 5.1 pg/ml, and eotaxin, 3 pg/ml.

Preparation of Nuclear Extracts

The left lungs were frozen. Nuclear extracts were prepared from frozen lung tissue using a Cellytic nuclear extraction kit (Sigma) according to the manufacturer's instructions [15]. The nuclear extracts were stored at -80°C until use.

NF- κ B ELISA

To evaluate the activation of NF- κ B, translocation of the p65 subunit into the nucleus was measured using a TransAM NF- κ B p65 Transcription Factor Assay kit (Active Motif Inc., Carlsbad, Calif., USA) according to the manufacturer's instructions [16]. The levels of activated NF- κ B were determined spectrophotometrically by measuring the absorbance at 450 nm and were expressed as optical density (OD450).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed using a Panomics™ EMSA Kit (Panomics Inc., Redwood City, Calif., USA). Nuclear proteins (5 µg) were incubated for 30 min at room temperature with the biotin-labeled oligonucleotide probe NF- κ B. Ten microliters of the reaction mixture were applied to denaturing 5% polyacrylamide gel electrophoresis for 35 min in TBE buffer (Bio-Rad Laboratories, Hercules, Calif., USA) and transferred to a nitrocellulose membrane using the iBlot™ GI Transfer System (Invitrogen, Carlsbad, Calif., USA). The blotted membrane was exposed to UV light in a UV Crosslinker (CX2000,

UVP, Upland, Calif., USA) for 3 min. Detection was performed following the manufacturer's instructions. The membranes were then incubated in Super Signal® (Pierce) and exposed to Fuji X-ray film for 5 min. For competition assays, unlabeled NF-κB was used.

Statistical Analysis

Data analysis was performed using Stat-View 5.0 (Abacus Concept Inc., Berkeley, Calif., USA). Experimental results were expressed as means $\pm$ SE. Experimental group results were compared using one-way analysis of variance (ANOVA). If statistical significance was identified by ANOVA, we used a Tukey-Kramer test to correct for multiple comparisons. For airway resistance measurements, two-way repeated-measures ANOVA was used, followed by a Tukey-Kramer test. Differences were considered to be statistically significant if *p* values were less than 0.05.

Results

Inhibition Characteristics of IMD-0354

The low-molecular-weight compound IMD-0354 inhibited 98.5% of NF-κB activity at a concentration of 10 μg/ml in HepG2 cells [median inhibition concentration (IC₅₀) 0.05 μM]. It also inhibited lipopolysaccharide-induced TNF-α (IC₅₀ 1.44 μM), IL-6 release (IC₅₀ 2.28 μM) in human peripheral blood mononuclear cells and the concanavalin A-induced release of IL-2 (IC₅₀ 0.71 μM), IL-4 (IC₅₀ 0.61 μM), IL-5 (IC₅₀ 0.38 μM) and IL-10 (IC₅₀ 0.23 μM). IMD-0354 did not inhibit other kinases (table 1), proteases or proteasome-related immune responses. IMD-0354 did not bind to enzymes or receptors related to these immune responses (table 1).

Inhibition of NF-κB by IMD-0354

To verify the inhibition of NF-κB activation by IMD-0354, we examined the activity of NF-κB in the nuclear fractions of lung tissues from OVA-sensitized mice in the presence or absence of IMD-0354. Nuclear proteins were extracted from the left lungs of mice as described in Materials and Methods. NF-κB activation in nuclear proteins was analyzed by NF-κB ELISA and EMSA.

As shown in figure 2A, the amounts of activated NF-κB in the OVA-sensitized/challenged mice (OVA-/OVA mice and OVA/CMC/OVA mice) were significantly increased compared to the control groups (OVA-/Saline mice and OVA/CMC/Saline mice). Administration of 20 mg/kg IMD-0354 significantly decreased the amount of activated NF-κB in the lungs. IMD-0354 at 5 mg/kg also significantly decreased NF-κB, but the magnitude of the decrease was lower than with 20 mg/kg IMD-0354 (OD450 of 0.50 $\pm$ 0.02 with 5 mg/kg vs. 0.43 $\pm$ 0.04 with 20 mg/kg).

Table 1. Percentage inhibition of kinases, enzymes and receptors by IMD-0354

	Species	Concentration, μM	Inhibition %
Kinase			
CAMKII	rat	100	33
ERK	human	100	34
MEK 1	rabbit	10	45
p38b	human	10	24
p38d	human	10	-37
p38g	human	10	0
PKA	bovine	100	-13
PKC	rat	300	6
EGFR	human	10	28
Enzyme			
Phospholipase A2	pig	300	62
Caspase 1	human	10	-14
Caspase 3	human	10	-10
Caspase 6	human	10	4
Caspase 7	human	10	-2
Caspase 8	human	10	3
MMP-1	human	10	-11
MMP-2	human	10	20
MMP-3	human	10	1
MMP-7	human	10	-6
MMP-9	human	10	-19
Receptor			
CCR1	human	10	-2
CCR2B	human	10	26
CCR4	human	10	-27
CCR5	human	10	-28
CXCR1	human	10	5
CXCR2	human	10	0
GR	human	10	8
IL-1R	mouse	10	15
IL-2R	mouse	10	-6
IL-6R	human	10	1
TNFR	human	10	-10

These data were provided by the Institute of Medicinal Molecular Design Inc.

To confirm the results of the NF-κB ELISA, EMSA was performed. The DNA-binding activity of NF-κB was markedly decreased in the nuclear extracts of lungs from OVA/IMD/OVA mice compared to OVA-/OVA and OVA/CMC/OVA mice (fig. 2B). The positive bands were attenuated upon the addition of an excess of unlabeled NF-κB oligonucleotide (fig. 2B).

These findings suggest that treatment with IMD-0354 inhibited NF-κB activation via blocking of the activity of IKKβ.

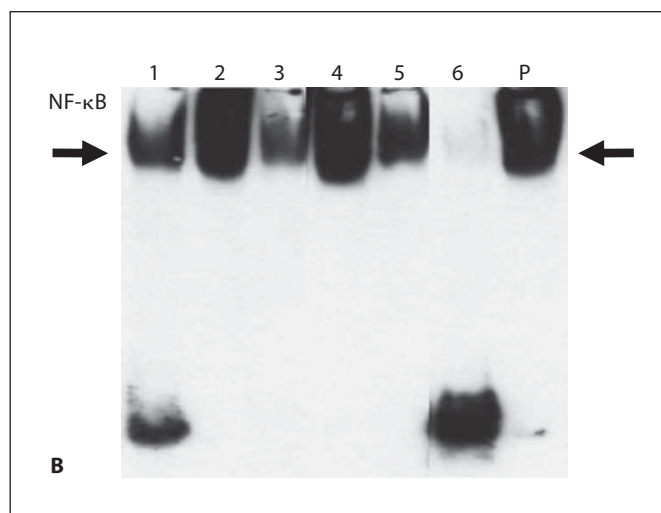
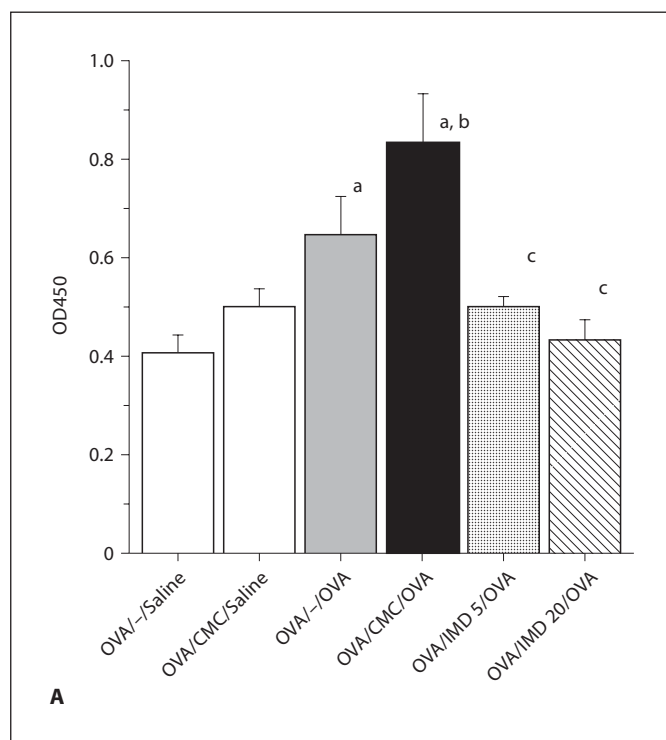


Fig. 2. Analysis of activation of NF- κ B by IMD-0354 in lungs of OVA-sensitized mice. **A** NF- κ B activation in lung tissue nuclear extracts of OVA-challenged mice (levels of the p65 subunit of the NF- κ B heterodimer). Results are shown as absorbance at 450 nm (OD450; mean $\pm$ SE) obtained from 3 different experiments. Numbers of mice in each group were as follows: OVA-/Saline mice, $n = 7$; OVA/CMC/Saline mice, $n = 9$; OVA-/OVA mice, $n = 9$; OVA/CMC/OVA mice, $n = 9$; OVA/IMD 5/OVA mice, $n = 10$; OVA/IMD 20/OVA mice, $n = 10$. ^a $p < 0.05$ compared to OVA-/Saline mice; ^b $p < 0.05$ compared to OVA/CMC/Saline

mice; ^c $p < 0.05$ compared to OVA/CMC/OVA mice. **B** DNA binding of NF- κ B in lung tissue nuclear extracts by EMSA. Lane 1: OVA-/Saline mice; lane 2: OVA-/OVA mice; lane 3: OVA-/OVA mice (unlabeled probe); lane 4: OVA/CMC/OVA mice; lane 5: OVA/CMC/OVA mice (unlabeled probe); lane 6: OVA/IMD 20/OVA; P: positive control. Competition experiments were performed by adding an excess of unlabeled oligonucleotide before the labeled probes (lanes 3 and 5). Results are representative of 3 separate experiments. Arrows indicate the NF- κ B band.

Effect of IMD-0354 on Allergy-Induced AHR

We assessed whether suppression of NF- κ B activity in the lung by IMD-0354 would also attenuate AHR. Penh was measured by unrestrained barometric whole-body plethysmography. There were significant differences between the curves for all groups [$F(5, 39) = 4.24$, $p = 0.004$]. Airway resistance was significantly increased in OVA-/OVA and OVA/CMC/OVA mice at 12.5 mg/ml Mch and all higher doses. The Penh measurements of OVA/IMD 5/OVA mice were similar to those of OVA/CMC/OVA mice (fig. 3A). IMD-0354 at 20 mg/kg significantly reduced airway resistance compared to that of OVA-/OVA and OVA/CMC/OVA mice at all concentrations of Mch (fig. 3A).

To confirm the Penh data, we also assessed R_L by restrained plethysmography. Repeated-measures two-way

ANOVA showed that the curves for all groups were significantly different [$F(3, 33) = 5.06$, $p < 0.005$; fig. 3B]. As shown in figure 3B, the percentage of baseline R_L for OVA/CMC/OVA mice was significantly increased with 6.3, 12.5 and 37.5 mg/ml Mch. IMD-0354 at 5 mg/kg decreased the R_L , although the magnitude of the decrease was low. IMD-0354 at 20 mg/kg reduced the airway resistance at all doses of Mch. At 6.3 and 37.5 mg/ml Mch, the percentage of baseline R_L for OVA/IMD 20/OVA mice was significantly decreased compared with OVA/CMC/OVA mice. These findings suggest that administration of IMD-0354 improved the AHR.

Pathological Features of OVA/IMD/OVA Mice

We assessed airway inflammation, eosinophils and mucus-producing cells in the airways of OVA-sensitized

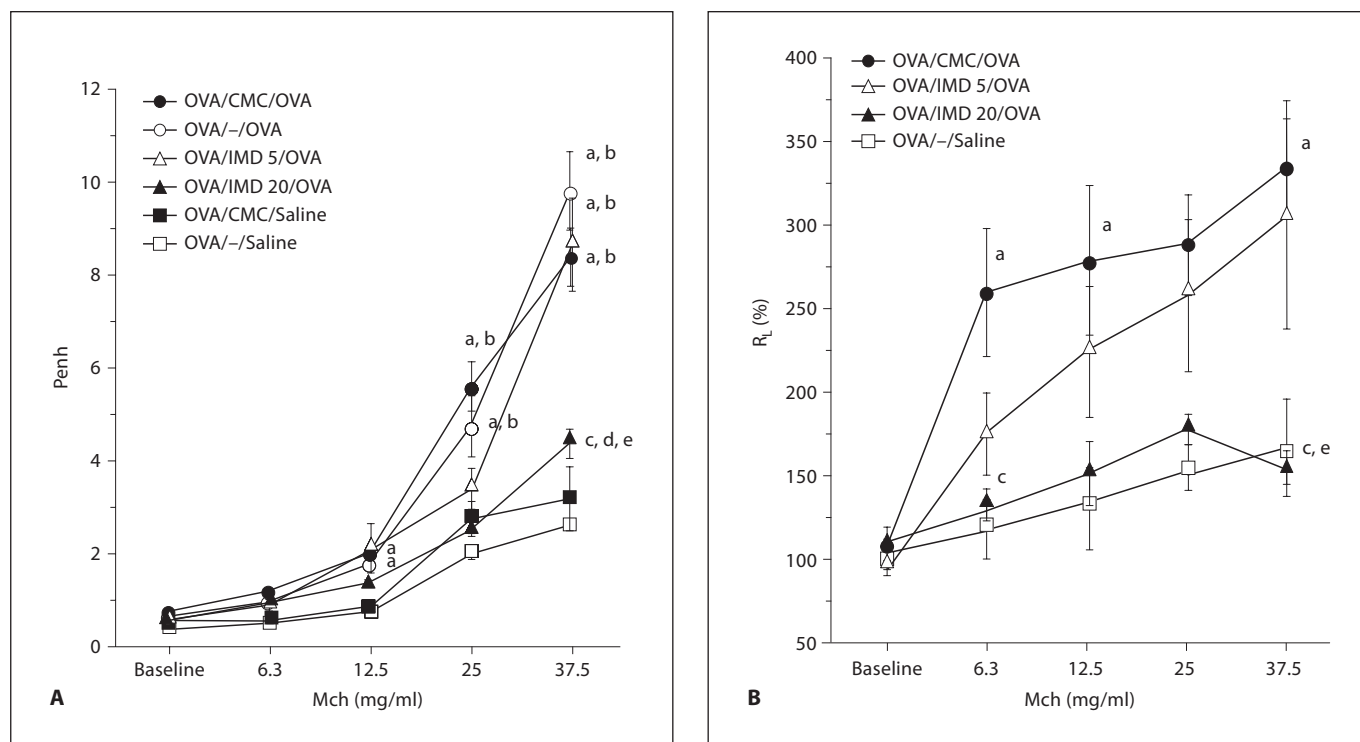


Fig. 3. Assessment of airway resistance after OVA challenge. Airway resistance was analyzed 48 h after the third daily aerosolized OVA challenge. **A** Penh measured by unrestrained whole-body plethysmography. Values were obtained in response to increasing concentrations of Mch, and results are means $\pm$ SE obtained from 3 different experiments. Numbers of mice in each group were as follows: OVA/CMC/OVA mice, $n = 9$; OVA/-/OVA mice, $n = 9$; OVA/IMD 5/OVA mice, $n = 8$; OVA/IMD 20/OVA mice, $n = 8$; OVA/CMC/Saline mice, $n = 6$; OVA/-/Saline mice, $n = 8$. **B** R_L measured by restrained whole-body plethysmography. Val-

ues were obtained with increasing concentrations of Mch and expressed as the percentage of baseline in OVA/-/Saline mice. Results are means $\pm$ SE obtained from 3 different experiments. Numbers of mice in each group were as follows: OVA/CMC/OVA mice, $n = 6$; OVA/IMD 5/OVA mice, $n = 5$; OVA/IMD 20/OVA mice, $n = 5$; OVA/-/Saline mice, $n = 6$. ^a $p < 0.05$ compared to OVA/-/Saline mice; ^b $p < 0.05$ compared to OVA/CMC/Saline mice; ^c $p < 0.05$ compared to OVA/CMC/OVA mice; ^d $p < 0.05$ compared to OVA/-/OVA mice; ^e $p < 0.05$ compared to OVA/IMD 5/OVA mice.

mice to evaluate the effect of IMD-0354 on this pathology. Left lung tissues of mice were collected and lung sections were prepared. The numbers of eosinophils and percentages of PAS-positive areas were analyzed using these sections as described in Materials and Methods.

IMD-0354 alone did not generate any changes in lung morphology (data not shown). Inflammatory cells were significantly increased in the subepithelia of the bronchi of OVA/-/OVA and OVA/CMC/OVA mice, but were significantly decreased in OVA/IMD 20/OVA mice (fig. 4A–F). In the Luna-stained sections, infiltrating eosinophils in the subepithelium were increased in OVA/-/OVA and OVA/CMC/OVA mice compared to OVA/-/Saline mice (fig. 4G–J). Administration of 20 mg/kg IMD-0354 led to a significant reduction of eo-

sophil infiltration, while 5 mg/kg IMD-0354 only partially reduced eosinophil infiltration (fig. 4K–L). As shown in figure 4S, treatment with 20 mg/kg IMD-0354 significantly decreased the numbers of infiltrating eosinophils.

Regarding mucus-producing cells, bronchial epithelial cells in OVA/-/OVA and OVA/CMC/OVA mice showed extensive PAS-positive staining, indicating the presence of mucus accumulation (fig. 4O, P). Both doses of IMD-0354 led to abrogation of mucus production (fig. 4Q, R). Statistical examination of these results showed that PAS-positive cells were increased in OVA/-/OVA and OVA/CMC/OVA mice, but were significantly decreased in OVA/IMD/OVA mice at both doses of IMD-0354 (fig. 4T).

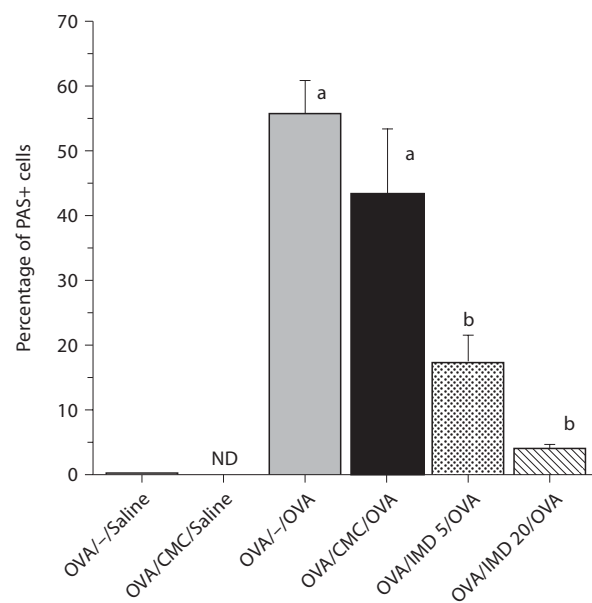
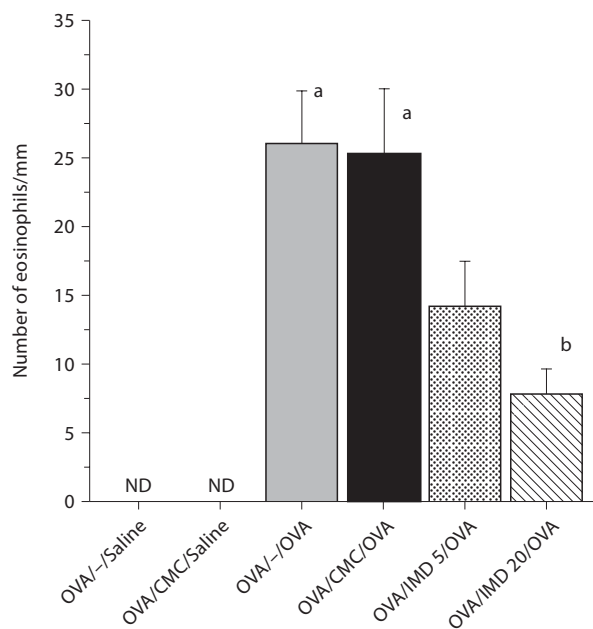
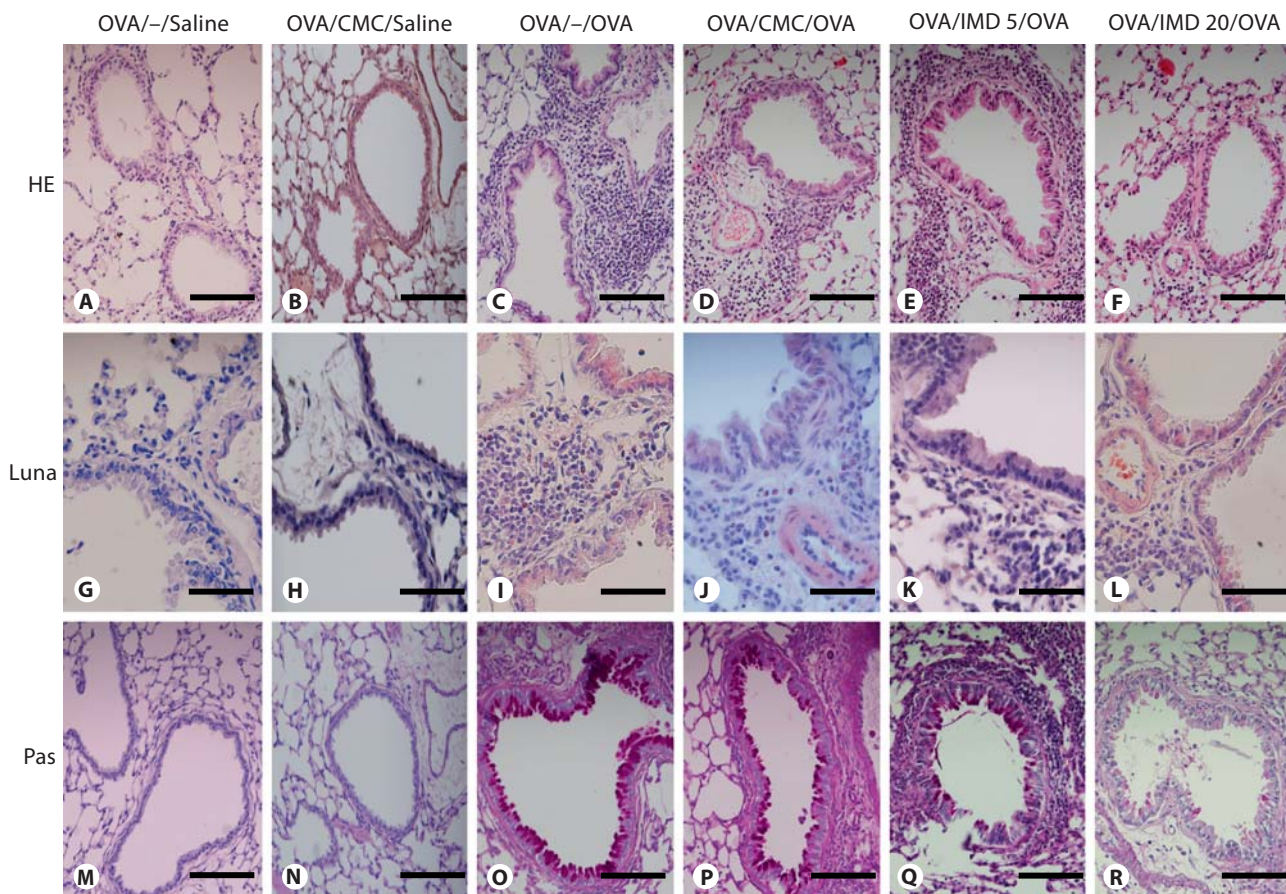


Table 2. Analysis of BALF

Mouse group	Total cells × 10 ⁴ /ml	Macrophages × 10 ⁴ /ml	Lymphocytes × 10 ⁴ /ml	Eosinophils × 10 ⁴ /ml
OVA/-/Saline (n = 10)	34.7 ± 5.8	32.5 ± 5.4	1.7 ± 0.6	0.1 ± 0.0
OVA/CMC/Saline (n = 9)	33.0 ± 5.3	29.7 ± 4.6	2.3 ± 0.5	0.0 ± 0.0 ^c
OVA/-/OVA (n = 11)	69.0 ± 10.4	31.6 ± 4.7	4.8 ± 0.6 ^a	26.8 ± 5.6 ^{a, b}
OVA/CMC/OVA (n = 12)	72.8 ± 11.5 ^{a, b}	37.7 ± 6.1	5.9 ± 1.2	27.1 ± 6.7 ^{a, b}
OVA/IMD 5/OVA (n = 10)	41.7 ± 7.5	26.6 ± 4.9	4.6 ± 0.9	8.6 ± 0.9 ^{c, d}
OVA/IMD 20/OVA (n = 12)	39.7 ± 4.9 ^c	29.3 ± 3.8	4.1 ± 0.8	6.0 ± 1.3 ^{c, d}
	IL-4, pg/ml	IL-13, pg/ml	Eotaxin, pg/ml	
OVA/-/Saline (n = 10)	12.2 ± 2.2	4.0 ± 1.3	4.7 ± 0.7	
OVA/CMC/Saline (n = 9)	6.9 ± 1.5	6.4 ± 1.2	0.9 ± 2.4	
OVA/-/OVA (n = 11)	14.1 ± 3.3	21.8 ± 6.6 ^a	9.0 ± 2.2	
OVA/CMC/OVA (n = 12)	12.6 ± 2.4	24.6 ± 6.5 ^a	12.5 ± 3.2 ^a	
OVA/IMD 5/OVA (n = 10)	11.7 ± 1.0	17.8 ± 3.3	5.8 ± 3.3	
OVA/IMD 20/OVA (n = 12)	11.9 ± 3.4	7.2 ± 1.1 ^c	3.1 ± 1.0 ^c	

Total cells, macrophages, lymphocytes, eosinophils and concentrations of cytokines in BALF recovered from the mice were analyzed. BALF was collected 72 h following the last antigen challenge, and differential cell counts were performed. Cytokine concentrations in the BALF were analyzed by ELISA. Results are means ± SE obtained from 6 different experiments.

^a p < 0.05 compared to OVA/-/Saline mice; ^b p < 0.05 compared to OVA/CMC/Saline mice; ^c p < 0.05 compared to OVA/CMC/OVA mice; ^d p < 0.05 compared to OVA/-/OVA mice.

BALF Cell Analysis and Cytokine/Chemokine Concentrations

Concomitant with the histological assessment, airway inflammation was evaluated using the BALF. BALF samples were collected and cell classifications and measurements of cytokine concentrations were performed as described in Materials and Methods. As shown in table 2, the total cell counts in the BALF recovered from OVA/CMC/OVA mice were significantly higher than those in

the BALF samples from the 2 groups of control mice. This difference was mainly due to a dramatic increase in the proportions of eosinophils in the BALF from asthmatic mice. Treatment with IMD-0354 (5 mg/kg) had no significant effect on total cells. Augmentation of eosinophils was decreased in OVA/IMD/OVA mice at both doses of IMD-0354. There were no differences in macrophages or lymphocytes in any of the groups. In addition, the IL-13 and eotaxin concentrations in the BALF of OVA/IMD 20/OVA mice were both significantly lower than those of OVA/-/OVA and OVA/CMC/OVA mice. In contrast, treatment with high-dose IMD-0354 did not affect the levels of IL-4 in BALF. IL-5, IFN- γ , IL-12, IL-1 β and TNF- α were not detected.

Effects of IMD-0354 on Chemokine and T Cell Cytokine Levels in Lung Homogenates

We examined the concentrations of all the cytokines listed above in lung homogenates, as some of them were not detected in the BALF. Supernatants of lung homogenates were prepared as described in Materials and Methods, and cytokine concentrations were measured by ELISA. As shown in figure 5, the levels of IL-5 and IL-13 produced in OVA/CMC/OVA mice were higher

Fig. 4. Histopathology of lungs from OVA-sensitized/challenged mice. Results are representative of 6 experiments. **A–F** Lung sections stained with HE. Original magnification: ×20. Bars = 100 μ m. **G–L** Lung sections stained by a modified Luna method. Original magnification: ×40. Bars = 50 μ m. **M–R** Lung sections stained with PAS. Original magnification: ×20. Bars = 100 μ m. **S, T** Numbers of infiltrated eosinophils (**S**) and percentage of mucus-producing cells in the total area of bronchial epithelial cells (**T**). Results are means ± SE obtained from 3 different experiments. Numbers of mice in each group were as follows: OVA/-/Saline mice, n = 6; OVA/CMC/Saline mice, n = 7; OVA/-/OVA mice, n = 8; OVA/CMC/OVA mice, n = 10; OVA/IMD 5/OVA mice, n = 8; OVA/IMD 20/OVA mice, n = 10. ^a p < 0.05 compared to OVA/-/Saline mice; ^b p < 0.05 compared to OVA/CMC/OVA mice. ND = Not determined.

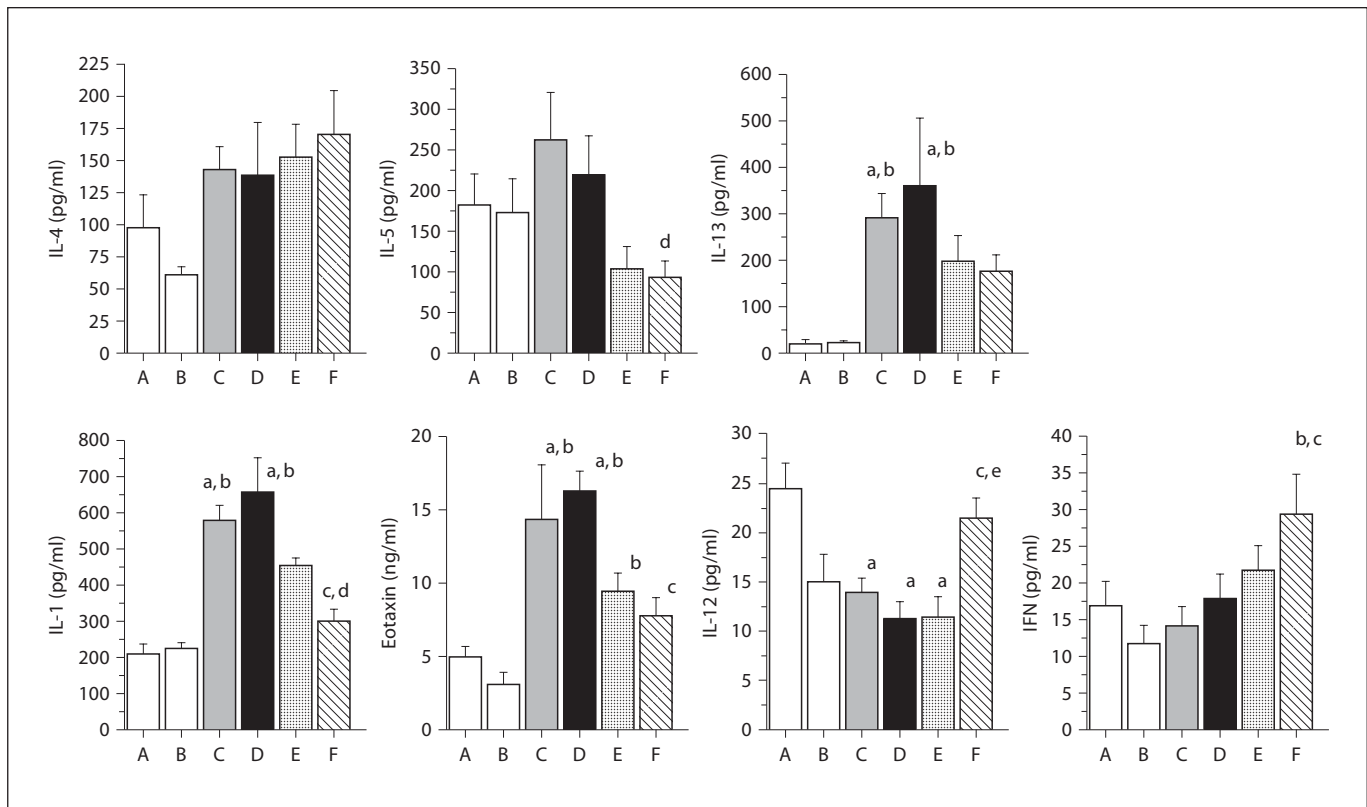


Fig. 5. Cytokines and chemokines in supernatants of lung homogenates. Lung tissue was harvested from 6 groups of mice on day 20. Supernatants of lung homogenates were collected and cytokine concentrations in them were analyzed by ELISA. Results are means $\pm$ SE obtained from 6 different experiments. A: OVA/-/Saline mice, n = 10; B: OVA/CMC/Saline mice, n = 9; C: OVA/-/

OVA mice, n = 11; D: OVA/CMC/OVA mice, n = 12; E: OVA/IMD 5/OVA mice, n = 10; F: OVA/IMD 20/OVA mice, n = 12. ^a p < 0.05 compared to OVA/-/Saline mice; ^b p < 0.05 compared to OVA/CMC/Saline mice; ^c p < 0.05 compared to OVA/CMC/OVA mice; ^d p < 0.05 compared to OVA/-/OVA mice; ^e p < 0.05 compared to OVA/IMD 5/OVA mice.

than in OVA/CMC/Saline mice. IMD-0354 at 20 mg/kg decreased IL-5 and IL-13 production. However, the IL-4 concentrations in the whole lungs did not significantly differ among the groups of mice. Eotaxin and IL-1 β concentrations in the lungs of OVA/IMD 20/OVA mice were significantly lower than in those of OVA/-/OVA and OVA/CMC/OVA mice. For IL-5, IL-13, eotaxin and IL-1 β , inhibitory effects on cytokine production were observed with 5 mg/kg IMD-0354, but the magnitude of the effects was lower than with 20 mg/kg IMD-0354. TNF- α was not detected in the lung homogenate samples.

IL-12 and IFN- γ concentrations in the lung homogenates of OVA/CMC/OVA mice were significantly lower than in those of OVA/CMC/Saline mice. Th1 cytokine levels in OVA/IMD 20/OVA mice were higher than in OVA/CMC/OVA mice. IMD-0354 at 5 mg/kg did not affect the Th1 cytokine concentrations.

Effects of IMD-0354 on Serum OVA-Specific IgE

A previous report showed that IgE production potentiated allergic airway inflammation and AHR in a mouse model of asthma [17]. Thus, we determined the serum levels of OVA-specific IgE in the presence or absence of IMD-0354. Blood was collected after the mice were sacrificed, and IgE concentrations were measured by ELISA. OVA challenge induced a significant increase in the serum levels of OVA-specific IgE in OVA/-/OVA and OVA/CMC/OVA mice compared with the levels in both groups of control mice (fig. 6A). The serum levels of OVA-specific IgE were significantly decreased in OVA/IMD 20/OVA mice compared with those in OVA/CMC/OVA mice. Only a partial decrease in serum IgE concentration was observed with 5 mg/kg IMD-0354.

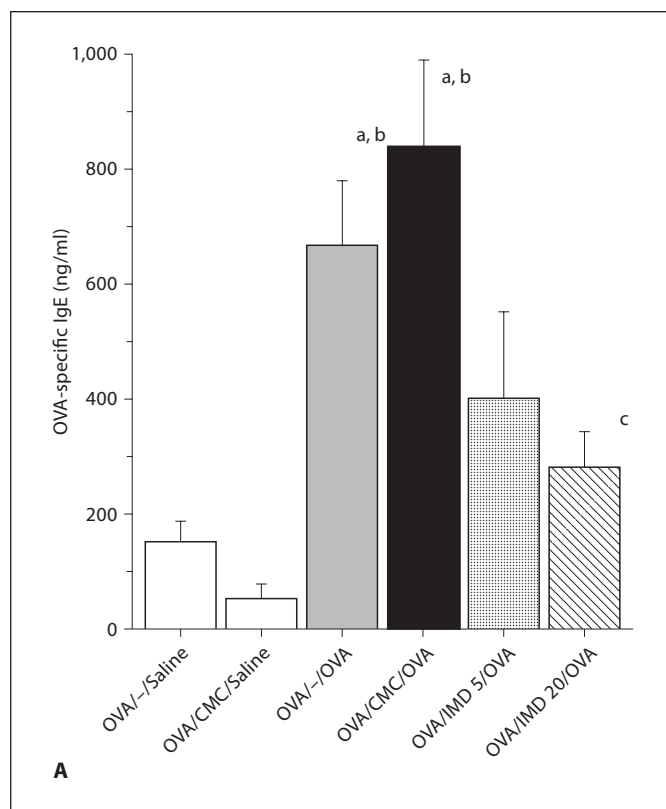
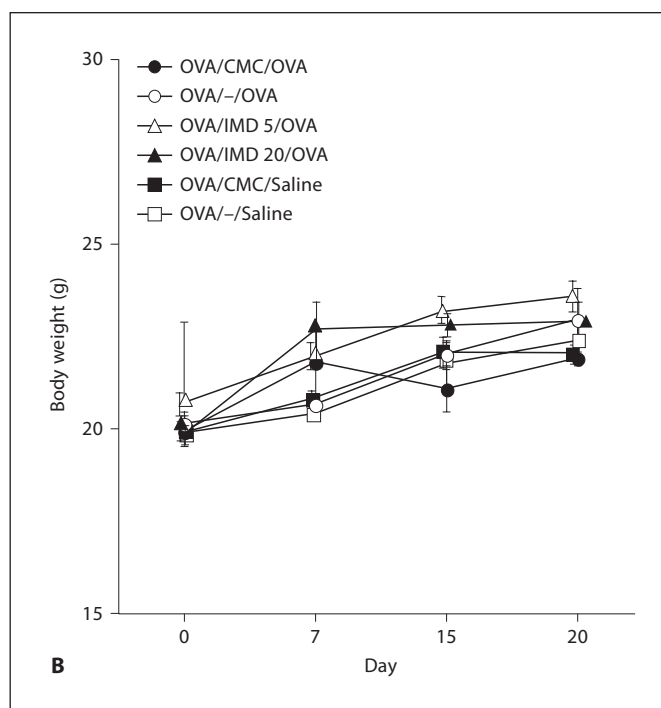


Fig. 6. A OVA-specific IgE concentration in the serum. Blood was collected after the mice were sacrificed, and the IgE concentration in serum was measured by ELISA. Results are means $\pm$ SE obtained from 6 different experiments. Numbers of mice in each group were as follows: OVA-/Saline mice, n = 10; OVA/CMC/Saline mice, n = 9; OVA-/OVA mice, n = 11; OVA/CMC/OVA mice, n = 12; OVA/IMD 5/OVA mice, n = 10; OVA/IMD 20/OVA mice, n = 12. ^a p < 0.05 compared to OVA-/Saline mice; ^b p < 0.05 compared to OVA/CMC/Saline mice; ^c p < 0.05 compared to OVA/CMC/OVA mice.



B Changes in body weight of OVA-sensitized/challenged mice. Body weight was determined on days 0, 7, 15 and 20. Results are means $\pm$ SE obtained from 3 different experiments. Numbers of mice in each group were as follows: OVA/CMC/OVA mice, n = 6; OVA-/OVA mice, n = 6; OVA/IMD 5/OVA mice, n = 10; OVA/IMD 20/OVA mice, n = 6; OVA/CMC/Saline mice, n = 6; OVA-/Saline mice, n = 6.

Effect of IMD-0354 Administration on Body Weight

In this model, administration of IMD-0354 was effective for suppression of allergic inflammation in the lung. We also examined the change in the body weight of the mice as an index of an adverse event.

The body weights of the mice were determined on days 0, 7, 15 and 20. There were no differences among the 7 groups of mice on day 7 (OVA-/Saline, 20.7 $\pm$ 0.4 g; OVA/CMC/Saline, 20.8 $\pm$ 0.2 g; OVA-/OVA, 20.5 $\pm$ 0.2 g; OVA/CMC/OVA, 22.3 $\pm$ 1.0 g; OVA/IMD 5/OVA, 21.3 $\pm$ 0.3 g; OVA/IMD 20/OVA, 22.6 $\pm$ 0.8 g; fig. 6B). By day 20, there was a similar increase in body weight in all groups of mice (OVA-/Saline, 22.1 $\pm$ 1.5 g; OVA/CMC/Saline, 22.0 $\pm$ 0.3 g; OVA-/OVA, 22.8 $\pm$ 0.5 g; OVA/CMC/OVA, 22.4 $\pm$ 0.2 g; OVA/IMD 5/OVA, 23.0 $\pm$ 0.4 g; OVA/IMD 20/OVA, 22.8 $\pm$ 0.5 g; fig. 6B).

Discussion

In this study, we demonstrated that a novel IKK β inhibitor, IMD-0354, effectively ameliorated AHR, eosinophil infiltration, mucus production and cytokine production in a mouse model of allergic inflammation.

IMD-0354 is a newly synthesized low-molecular-weight compound that specifically inhibits IKK β , which induces the inhibition of NF- κ B activation only in inflammatory conditions. We showed that IMD-0354 does not inhibit other kinases, proteases or proteasome-related immune responses (table 1). Preliminary data have shown that IKK α -dependent p100 processing is not affected by IMD-0354. Onai et al. [9] demonstrated that IMD-0354 inhibited NF- κ B activation in HepG2 cells in which a constitutively active mutant IKK β is expressed.

These findings suggested that IMD-0354 specifically inhibits IKK β in the intact IKK complex, leading to inhibition of active NF- κ B.

NF- κ B activation leads to the production of various inflammatory cytokines, suggesting that it is an ideal target molecule for reducing inflammation. Indeed, previous reports showed that inhibition of NF- κ B improved allergic airway inflammation and AHR via decreased production of cytokines such as IL-5, IL-13 and eotaxin in animal models of asthma [18, 19]. These effects were caused by the direct inhibition of NF- κ B, which is functional under normal conditions. Since IMD-0354 is able to target IKK β in the IKK complex only under inflammatory conditions caused by a proinflammatory cytokine such as TNF- α , IMD-0354 does not affect NF- κ B function under normal conditions. These observations suggest that IKK β targeting by IMD-0354 could be more useful, and safer, for the treatment of bronchial asthma than drugs that directly inhibit NF- κ B. As a caution, however, it must be noted that recent reports show that NF- κ B activation contributes to host defense against bacteria or viruses [20, 21]. IKK β is related to host defense by NF- κ B activation [20]. However, other reports show that the signaling for NF- κ B activation for host defense occurs not only along the IKK β -NF- κ B pathway, but also along the IKK α , ck2 and p38 pathways [22]. Although it is possible that inhibition of IKK β by IMD-0354 does not affect host defense, it remains unclear. Thus, further investigations are necessary to clarify the effects of IMD-0354 on these actions of NF- κ B.

In previous reports of IKK β inhibition in a mouse model of asthma, it was found that TPCA-1 [7] and Compound A [8] prevented allergic pulmonary inflammation and a Th2 response. These results are confirmed by our present findings with IMD-0354. Th2 cytokines, such as IL-5 and IL-13, have an important role in the pathogenesis of asthma [23, 24]. Eotaxin has been shown to be a specific chemoattractant for eosinophils and Th2 cells [25]. The combined decreases in these cytokines and chemokines in the airways by IMD-0354 treatment may lead to a significant attenuation of hyperresponsiveness, eosinophil infiltration and mucus production.

Interestingly, 5 mg/kg IMD-0354 did not significantly ameliorate hyperresponsiveness, although NF- κ B activation was significantly decreased by this dose of IMD-0354. In the present findings, the magnitudes of the inhibitory effects of 5 mg/kg IMD-0354 on NF- κ B activation and cytokine production were lower than for 20 mg/kg IMD-0354. From these observations, it seems likely that 5 mg/kg IMD-0354 could not sufficiently over-

come the production of inflammatory cytokines, resulting in a failure to significantly ameliorate AHR. In contrast, 20 mg/kg IMD-0354 improved all parameters of allergic inflammation and did not affect the general physiology, e.g. body weight. The results in a bleomycin-induced pulmonary fibrosis model also showed that 20 mg/kg IMD-0354 did not affect the survival of wild-type mice [11]. These findings suggest that 20 mg/kg IMD-0354 is an appropriate and safe dose for the inhibition of allergic inflammation.

Ziegelbauer et al. [8] also showed that an IKK β inhibitor, Compound A, reduced not only Th2 cytokine production but also IL-12 production. Although the reason is not clear, it is possible that Compound A might affect the NF- κ B pathway of Th1 cytokine production. The effect of TPCA-1 on Th1 cytokine regulation was also unclear [7]. In contrast to these previous reports, our present findings showed that IL-12 and IFN- γ production were restored in OVA/IMD/OVA mice. It is likely that inhibition of the Th2 cell response by IMD-0354 led to the restoration of the Th1 response through an NF- κ B-independent pathway. These findings suggest that IMD-0354 did not directly affect the pathway of Th1 cytokine production in allergic inflammatory conditions. Previous reports in mice showed that restoration of the Th1/Th2 balance in localized areas prevented AHR and reduced lung eosinophilia following allergen challenge in sensitized mice [26, 27]. IFN- γ inhibits IgE production in OVA-sensitized mice [26] and TGF- β production in bronchial epithelial cells [28] and fibroblasts [29]. In light of these reports, restoration of the Th1 cytokines by IMD-0354 may have a synergistic role in the improvement of allergic inflammation.

It should also be noted that IgE production was reduced by IMD-0354 treatment in our model. Previous reports of IKK β inhibitors [7, 8] did not clarify the effects on IgE production, and an NF- κ B decoy was found not to improve the IgE production [19]. IgE plays an important role in the development of allergic airway inflammation and AHR in the rat [17]. IgE is produced by B cells, and IgE aggregation stimulates mast cells, causing an early allergic response in the airway. IMD-0354 was found to inhibit the synthesis of IgE by B cells in a dose-dependent manner [30], suggesting one mechanism for inhibition of IgE production by IMD-0354 in our model. Clinically, an anti-IgE antibody has been shown to be effective in patients with poorly controlled, severe persistent allergic asthma [31], supporting the idea that reduction of IgE may be a useful therapy for patients with refractory asthma. Therefore, control of IgE production by IMD-0354

may also be an effective strategy for the management of allergic inflammation.

In conclusion, IMD-0354 has the potential to improve allergic airway inflammation and AHR. These findings suggest that IMD-0354 will be a candidate drug for the treatment of patients with asthma. To clarify the effects of IMD-0354 in the asthmatic patient, additional studies will be necessary.

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