



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

GS143, an I κ B ubiquitination inhibitor, inhibits allergic airway inflammation in mice [☆]

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ARTICLE INFO

Article history:

Received 8 July 2008

Available online xxxxx

Keywords:

Asthma

Th2 cells

Eosinophils

I κ B

Ubiquitination

ABSTRACT

Asthma is characterized by airway inflammation with intense eosinophil infiltration and mucus hyperproduction, in which antigen-specific Th2 cells play critical roles. Nuclear factor- κ B (NF- κ B) pathway has been demonstrated to be essential for the production of Th2 cytokines and chemokines in the airways in murine asthma models. In the present study, we examined the effect of GS143, a novel small-molecule inhibitor of I κ B ubiquitination, on antigen-induced airway inflammation and Th2 cytokine production in mice. Intranasal administration of GS143 prior to antigen challenge suppressed antigen-induced NF- κ B activation in the lung of sensitized mice. Intranasal administration of GS143 also inhibited antigen-induced eosinophil and lymphocyte recruitment into the airways as well as the expression of Th2 cytokines and eotaxin in the airways. Moreover, GS143 inhibited antigen-induced differentiation of Th2 cells but not of Th1 cells *in vitro*. Taken together, these results suggest that I κ B ubiquitination inhibitor may have therapeutic potential against asthma.

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Asthma is characterized by a chronic airway inflammation with intense eosinophil and lymphocyte infiltration, mucus hyperproduction, and airway hyper-responsiveness to a variety of stimuli [1]. It is now generally accepted that these pathognomonic features of asthma are orchestrated by antigen-specific Th2 cells and their cytokines such as IL-4, IL-5, and IL-13 [2–4]. Importantly, it has been demonstrated that nuclear factor- κ B (NF- κ B) is essential for the production of Th2 cytokines as well as a number of chemokines in the airways in murine asthma models [5]. It has also been shown that NF- κ B is required for the differentiation of Th2 cells by inducing the expression of GATA3 [6], a master regulator of Th2 cell differentiation [7]. The importance of NF- κ B in the pathogenesis of asthma is further underscored by the fact that the airways of asthmatic patients exhibited the enhanced NF- κ B activity, especially in airway epithelial cells and macrophages [8,9]. These findings suggested that NF- κ B pathway plays pivotal roles in the

development of asthma and thus that the inhibition of NF- κ B pathway might be an effective therapeutic approach to asthma.

NF- κ B is typically a heterodimer that consists of p65 and p50 [10,11]. NF- κ B is normally retained in the cytoplasm by its association with the inhibitory molecule, I κ B. In the classical pathway of NF- κ B activation, inflammatory stimuli lead to the activation of I κ B kinase (IKK), which phosphorylates serine residues of I κ B protein. Phosphorylated I κ B is recognized by ubiquitin-conjugated machinery, leading to the poly-ubiquitination and subsequent degradation by proteasome. The proteolysis of I κ B allows NF- κ B to translocate to the nucleus where NF- κ B activates transcription of a number of proinflammatory cytokines and chemokines important for inducing acute inflammatory responses. These findings suggest that an inhibitor of I κ B ubiquitination may be an efficient tool for the suppression of NF- κ B activation and subsequent NF- κ B-mediated inflammatory responses. However, it remains to be determined whether an I κ B ubiquitination inhibitor has therapeutic potential against allergic diseases such as asthma.

By establishing time-resolved fluorescence resonance energy transfer (TR-FRET) assay to identify a compound that inhibits the ubiquitination of I κ B, we identified a small-molecule inhibitor which specifically inhibited I κ B ubiquitination [12]. This compound, GS143, specifically inhibits the ubiquitination of I κ B and suppresses NF- κ B activation and transcription of target genes without affecting cell survival and proliferation [12]. Here, we examined the therapeutic

[☆] This work was supported in part by the grants from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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potential of GS143 on allergic airway inflammation using a murine model of asthma. We found that the intranasal administration of GS143 suppressed antigen-induced NF- κ B activation in the lung of sensitized mice. In concert with this finding, the intranasal administration of GS143 suppressed antigen-induced eosinophil recruitment into the airways, mucus production, and Th2 cytokine and eotaxin expression in the airways. Further investigations demonstrated that GS143 suppressed Th2 cell but not Th1 cell differentiation *in vitro*. These results highlight the therapeutic potential of the selective inhibitor of I κ B ubiquitination against allergic airway inflammation.

Materials and methods

Mice. BALB/c mice were purchased from Charles River Laboratories (Atsugi, Japan). DO11.10 mice on a BALB/c background were described previously [13]. All mice were housed in microisolator cages under specific pathogen-free conditions and animal procedures used in this study conformed to NIH guidelines and were approved by the Chiba University Animal Care and Use Committee.

Antigen-induced allergic inflammation in the airways. BALB/c mice (age 7–8 weeks) were immunized intraperitoneally twice with 50 μ g of ovalbumin (OVA) in 4 mg of aluminum hydroxide at a 2-week interval. Two weeks after the last immunization, the sensitized mice were challenged with aerosolized OVA (50 mg/ml) dissolved in saline for 20 min through a DeVilbiss 646 nebulizer (DeVilbiss Corporation, Somerset, PA) [14]. As a control, saline alone was administered by the nebulizer. When indicated, GS143 (4-[3-benzyl-4-[5-(2-fluorophenyl)-furan-2-ylmethylene]-5-oxo-4,5-dihydropyrazol-1-yl]-benzoic acid [12] (16 or 32 μ g/body) (provided by GeneCare Research Institute Co. Ltd. (Kanagawa, Japan)) or vehicle control (dimethyl sulfoxide) diluted with 50 mM Tris–HCl (pH 7.4) containing 50 mM cysteine was administered in a volume of 20 μ l by intranasal instillation to sensitized mice 2 h before OVA or saline inhalation. The lung sections and the number of eosinophils, neutrophils, and lymphocytes recovered in the bronchoalveolar lavage fluid (BALF) were evaluated as described previously [15].

Nuclear accumulation of NF- κ B p65. OVA-sensitized BALB/c mice were given GS143 (32 μ g/body) or vehicle and 2 h later, mice were challenged with the inhaled OVA or saline as described above. One or 2 h after the OVA inhalation, nuclear extracts were prepared from whole lung using CellLytic NuCLEAR Extraction kit (Sigma, St. Louis, MO) according to the manufacturer's instruction. DNA-binding activity of NF- κ B p65 in the nuclear extracts was determined by Transfactor NF- κ B chemiluminescent kit (BD Bioscience, San Diego, CA). In brief, nuclear extracts from whole lung (5 μ g) were added to wells coated with NF- κ B consensus oligonucleotides and incubated for 1 h at room temperature. After washing, the wells were incubated with rabbit polyclonal anti-NF- κ B p65 antibody, followed by anti-rabbit IgG HRP and then chemiluminescent substrate mixture. Chemiluminescent intensities were measured with Arvo 1420 multilabel counter (Wallac, Gaithersburg, MD).

ELISA. The levels of IL-5 and IL-13 in the BALF and of IFN- γ and IL-4 in the culture supernatants were determined by enzyme immunoassay according to the manufacture's instruction (R&D Systems, Minneapolis, MN). The detection limits of these assays were 7 pg/ml for IL-13 and 20 pg/ml for IL-4, IL-5, and IFN- γ .

Real-time PCR analysis. Expression of eotaxin-1 and eotaxin-2 mRNA was determined by real-time quantitative PCR with a standard protocol on an ABI PRISM 7300 instrument (Applied Biosystems, Foster City, CA) using a SYBR green reagent (Power SYBR Green PCR Master Mix, Applied Biosystems) [15]. The levels of each mRNA were normalized to the levels of β -actin mRNA.

Antigen-induced T helper cell differentiation. Splenocytes from DO11.10 mice (2×10^6 /ml) were stimulated with OVA323–339 peptide (0.1 μ g/ml) in a 48-well microtiter plate at 37 $^{\circ}$ C for 5 days [16]. GS143 (10 or 20 μ M) or vehicle was added to the culture 30 min

before stimulation with OVA323–339 peptide. Where indicated, IL-12 (10 ng/ml; R&D Systems Inc.) was added to polarize toward Th1 cells (Th1-polarizing conditions), and IL-4 (10 ng/ml; R&D Systems Inc) and anti-IFN- γ antibody (20 μ g/ml; XMG1.2, BD PharMingen) were added to polarize Th2 cells (Th2-polarizing conditions). Intracellular cytokine staining for IFN- γ and IL-4 was performed with anti-IFN- γ allophycocyanin (APC) (XMG1.2, BD PharMingen) and anti-IL-4 phycoerythrin (PE) (BVD4-1D11, BD PharMingen) according to the manufacturer's instructions.

Data analysis. Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by the unpaired *t* test. *P* values <0.05 were considered significant.

Results

Intranasal administration of GS143 suppresses antigen-induced NF- κ B activation in the airways of sensitized mice

We first examined whether the intranasal administration of GS143 inhibited antigen-induced NF- κ B activation in the lung of sensitized mice. The basal levels of NF- κ B activity were detected even in the lung of vehicle-treated, saline-inhaled OVA-sensitized mice (Fig. 1A) and the intranasal administration of GS143 exhibited no effect on the basal NF- κ B activity (Fig. 1A). As expected, the levels of NF- κ B activity were significantly enhanced in vehicle-treated, OVA-sensitized mice at 2 h after OVA challenge (Fig. 1A). Intranasal administration of GS143 prior to the OVA challenge significantly suppressed antigen-induced NF- κ B activation almost to the basal levels ($n = 4$ mice in each group, $p < 0.01$) (Fig. 1A). These results indicate that the intranasal administration of GS143 efficiently inhibits antigen-induced NF- κ B activation in the lung.

GS143 suppresses antigen-induced airway inflammation and mucus production

Given that the intranasal administration of GS143 inhibits antigen-induced NF- κ B activation in the lung (Fig. 1A), we next examined whether the administration of GS143 affected antigen-induced inflammatory responses and mucus production in the airways. GS143 or vehicle was intranasally administered to OVA-sensitized mice and 2 h after the administration, the mice were challenged with OVA inhalation. Histological analysis of vehicle-treated, OVA-inhaled mice showed prominent peribronchial and perivascular inflammatory cell infiltration in the lung, which mainly consisted of lymphocytes and eosinophils (Fig. 1B). Importantly, intranasal administration of GS143 significantly inhibited antigen-induced airway inflammation in sensitized mice (Fig. 1B). In addition, PAS-stained lung sections revealed that goblet cell hyperplasia was also significantly inhibited by the intranasal administration of GS143 (Fig. 1B).

Consistent with the data obtained by histological analyses, the number of cells recovered from the BALF was significantly decreased in GS143-treated mice (32 μ g) as compared with those in vehicle-treated mice (Fig. 1C). Especially, the numbers of eosinophils and lymphocytes in the BALF were significantly decreased by GS143 treatment ($n = 4$ –7 mice in each group, $p < 0.01$) (Fig. 1C). By contrast, the numbers of macrophages in the BALF were not affected by GS143 treatment (data not shown). These results suggest that intranasal administration of GS143 significantly suppresses antigen-induced inflammatory responses and mucus production in the airways of sensitized mice.

GS143 suppresses antigen-induced Th2 cytokine and chemokine expression in the airways of sensitized mice

To determine the basis for GS143-mediated suppression of antigen-induced airway inflammation, we next evaluated cyto-

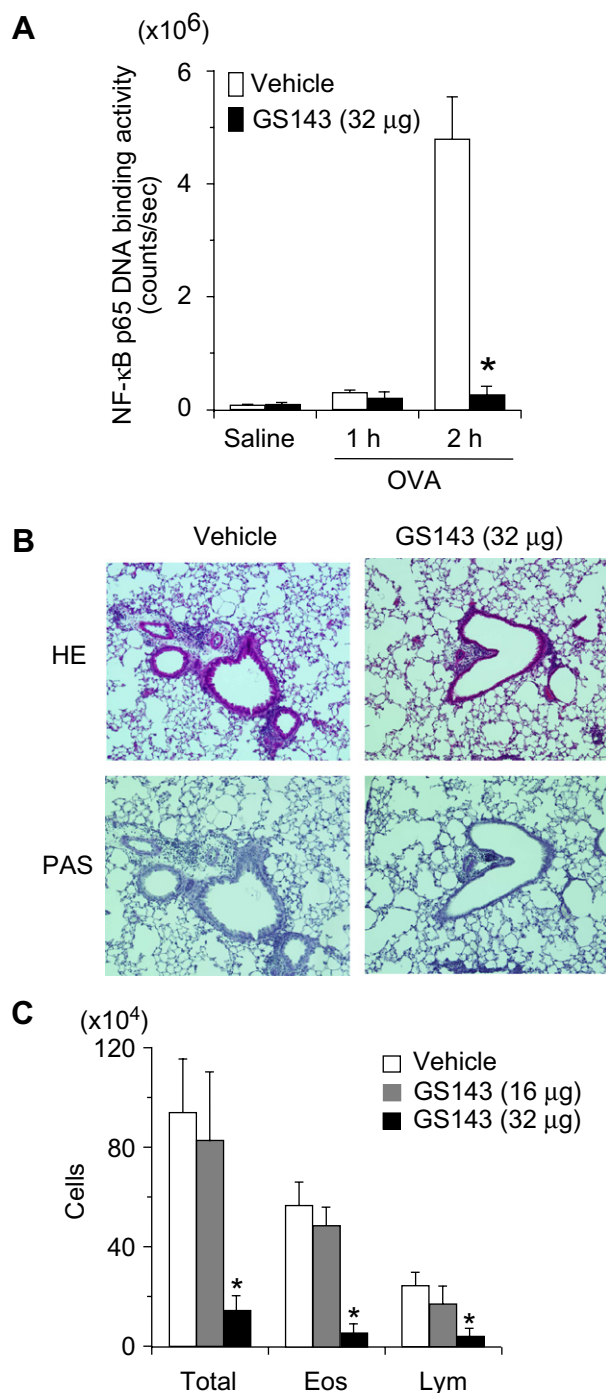


Fig. 1. GS143 suppresses antigen-induced NF- κ B activation in the lung and airway inflammation in sensitized mice. (A–C) OVA-sensitized mice were given intranasally with GS143 (16 or 32 μ g) or vehicle and 2 h later, these mice were challenged with inhaled OVA or saline (as a control). (A) Indicated times after the OVA inhalation, nuclear proteins were extracted from whole lungs and then assessed for NF- κ B p65 DNA-binding activity. Data are means $\pm$ SD for 4 mice in each group. * p < 0.01. (B) Forty-eight hours after the OVA challenge, the lungs were removed and histological analysis was performed. Representative photomicrographs of lung sections stained with HE and PAS are shown. n = 7 mice in each group. (C) Forty-eight hours after the OVA challenge, BALF were harvested and the numbers of total cells, eosinophils (Eos), and lymphocytes (Lym) in the BALF were determined. Data are means $\pm$ SD for 4–7 mice in each group. * p < 0.01.

kine levels in the BALF in GS143-treated mice. Consistent with the attenuated eosinophilic inflammation and mucus production in the airways (Fig. 1), the levels of IL-5 and IL-13 in the BALF were

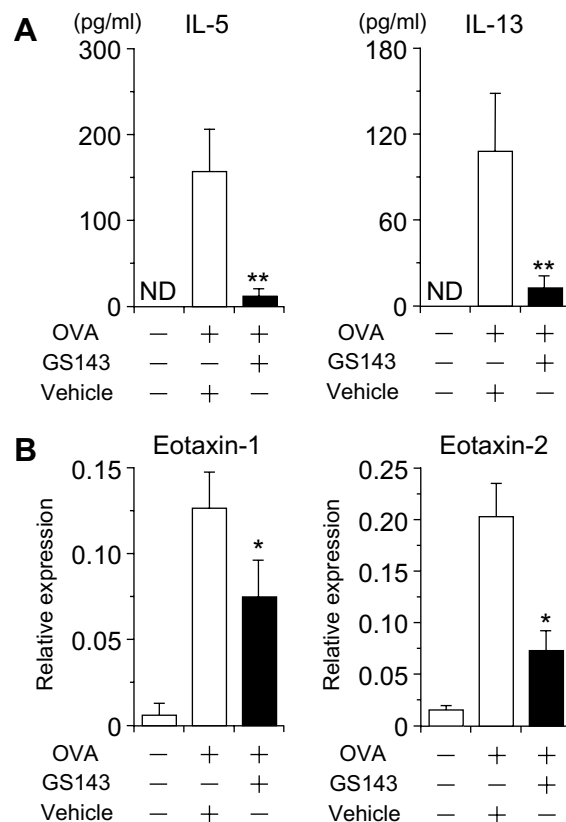


Fig. 2. GS143 suppresses antigen-induced Th2 cytokine and eotaxin production in the airways of sensitized mice. (A,B) OVA-sensitized mice were given intranasally with GS143 (32 μ g) or vehicle and 2 h later, these mice were challenged with inhaled OVA or saline. (A) Forty-eight hours after the OVA challenge, BALF were harvested and the levels of IL-5 and IL-13 in the BALF were determined. Data are means $\pm$ SD for 7 mice in each group. ** p < 0.01. (B) Forty-eight hours after the OVA challenge, total RNA was prepared from the lung tissues and real-time PCR analysis for eotaxin-1, eotaxin-2, and β -actin was performed. The levels of eotaxin-1 and eotaxin-2 were normalized to the levels of β -actin. Data are means $\pm$ SD for 5 mice in each group. * p < 0.05.

significantly decreased by GS143 treatment (n = 7 mice, p < 0.01) (Fig. 2A). The levels of eotaxin-1 and eotaxin-2, key chemokines inducing eosinophilic inflammation [17], were also significantly suppressed in GS143-treated mice as compared with those in vehicle-treated mice (n = 5 mice, p < 0.05) (Fig. 2B). These findings are consistent with a previous report demonstrating the reduced eotaxin-1 expression in NF- κ B p50-deficient mice [5]. These results indicate that the intranasal administration of GS143 suppressed allergic airway inflammation and mucus production in part through the suppression of Th2 cytokine and chemokine expression in the airways of sensitized mice.

GS143 inhibits Th2 cell but not Th1 cell differentiation

Because in vivo administration of GS143 inhibited allergic airway inflammation in which Th2 cells play central roles, we next examined the direct effect of GS143 on Th2 cell differentiation. CD4⁺ T cells from OVA-specific DO11.10 T cell receptor transgenic mice were stimulated with OVA peptide under Th1- or Th2-polarizing condition in the presence or absence of GS143 and 5 days later, IL-4 and IFN- γ production upon re-stimulation with anti-CD3 antibody were evaluated. As shown in Fig. 3A, whereas IFN- γ production in Th1-polarizing condition was not suppressed by GS143, IL-4 production in Th2-polarizing condition was markedly suppressed by GS143 in a dose-dependent manner (n = 4 mice).

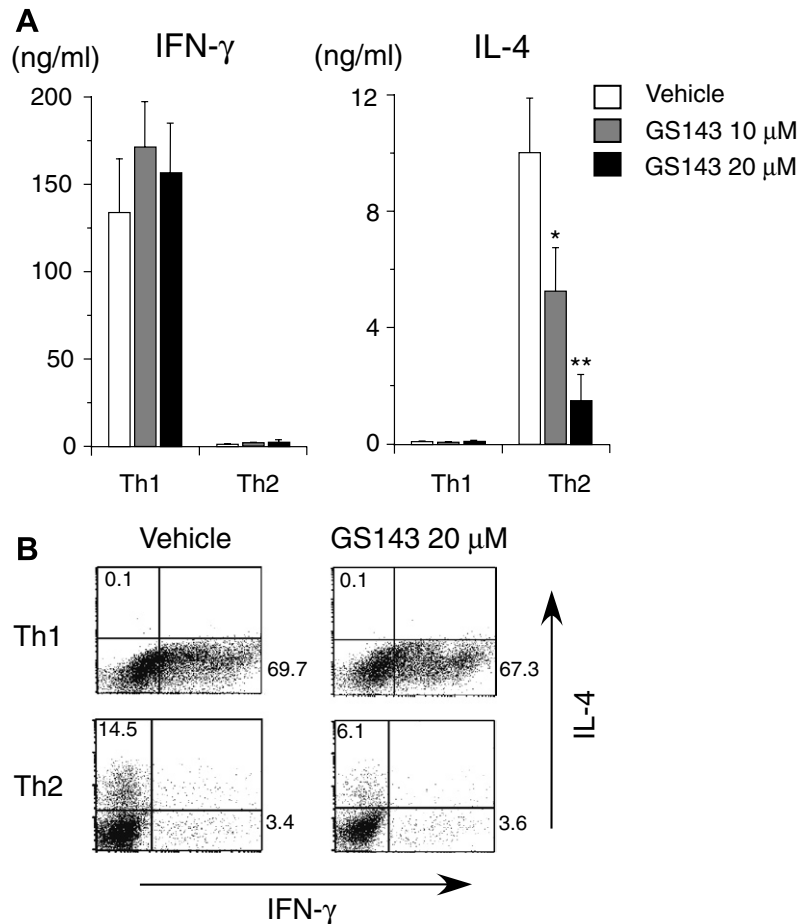


Fig. 3. GS143 inhibits Th2 cell differentiation. (A,B) Splenocytes from DO11.10 mice were stimulated with OVA 323–339 peptide under Th1- or Th2-polarizing condition for 5 days in the presence or in the absence of GS143 (10 or 20 μM). (A) Cells (2×10^5 /ml) were harvested from the culture and re-stimulated with plate-bound anti-CD3 antibody for 24 h. The levels of IFN-γ and IL-4 in the culture supernatants were determined by ELISA. Data are means $\pm$ SD for 4 mice in each group. * $p < 0.05$, ** $p < 0.01$. (B) Similar to (A), cells were re-stimulated with plate-bound anti-CD3 antibody for 6 h with monensin added for the final 4 h to prevent cytokine release. Intracellular cytokine profiles (IFN-γ vs. IL-4) were determined on CD4⁺ T cells. Shown were representative FACS profiles from 5 mice in each group.

The inhibitory effect of GS143 on Th2 cell differentiation was confirmed by intracellular cytokine staining at single cell levels (Fig. 3B). The proportion of IL-4 producing cells under Th2-polarizing condition was reduced by 58% by GS143. On the other hand, GS143 did not significantly inhibit Th1 cell differentiation under Th1-polarizing condition (Fig. 3B). GS143 did not significantly inhibit T cell proliferation, either (data not shown). Taken together, these results suggest that the inhibitory effect of GS143 on Th2 cytokine production may account in part for the effect of GS143 on allergic airway inflammation.

Discussion

In the present study, we show that GS143, a selective inhibitor of IκB ubiquitination, suppresses antigen-induced airway inflammation and Th2 cell differentiation in mice. We found that the intranasal administration of GS143 suppressed antigen-induced NF-κB activation in the airways of sensitized mice (Fig. 1). We also found that GS143 suppressed antigen-induced eosinophil and lymphocyte recruitment into the airways, Th2 cytokine and eotaxin production, and mucus production in the airways (Figs. 1 and 2). In vitro, GS143 suppressed antigen-induced Th2 cell differentiation (Fig. 3). Taken together, these results suggest that the inhibition of IκB ubiquitination could be a therapeutic approach against asthma.

We show that GS143 inhibits antigen-induced airway inflammation using a murine model of asthma (Fig. 1). To date, several lines of evidence have revealed that NF-κB pathway plays a pivotal role in Th2 cell differentiation and development of allergic airway inflammation [5,6]. It has also been demonstrated that the activation of NF-κB in the airway epithelial cells is required for inducing airway inflammation at the effector phase of allergic airway inflammation [18–20]. In accordance with these data, we found that intranasal administration of GS143 not only inhibited antigen-induced Th2 cytokine production and eosinophil recruitment into the airway but also suppressed eotaxin expression in the airways (Figs. 1 and 2). Given that the major eotaxin-producing cells in allergic airways were airway epithelial cells [21], it is suggested that one of target cells by intranasal administration of GS143 could be airway epithelial cells. Because both eotaxin-1 and eotaxin-2 are potent chemokines inducing eosinophil recruitment [17], the inhibition of eotaxin expression by GS143 administration could be involved in the attenuated eosinophil recruitment into the airways.

Interestingly, compared with a previous report in which NF-κB pathway was inhibited specifically in the airway epithelial cells [20], intranasal administration of GS143 showed marked inhibition of airway inflammation (Fig. 1), suggesting the presence of another target cells in GS143-mediated inhibition of airway inflammation in antigen-challenged mice. In this regard, we demonstrated that GS143 inhibits the differentiation of Th2

cells (Fig. 3), which play critical roles in inducing allergic airway inflammation [2–4]. Taken together, these results suggest that GS143 inhibits allergic airway inflammation by at least two mechanisms. First, GS143 inhibits the expression of eotaxin-1 and eotaxin-2 from airway epithelial cells. Second, GS143 attenuates antigen-induced Th2 cell activation in the airways. The combined inhibition may account for the strong effect of GS143 on eosinophilic airway inflammation.

Our data provide two important points for the future development of NF- κ B inhibitors for the treatment of inflammatory diseases including asthma. First, our data provide direct evidence that an inhibition of I κ B ubiquitination is effective to suppress NF- κ B activation in vivo. To date, most of the NF- κ B inhibitors target IKK proteins [22] and some of the inhibitors target proteasome [23,24]. To our knowledge, GS143 is the first compound that inhibits NF- κ B pathway through the suppression of I κ B ubiquitination [12]. From the viewpoint of the adverse effects, the high selectivity of GS143 on I κ B ubiquitination might have a great advantage for clinical application. Although we need to await further studies to clarify whether the inhibitor of I κ B ubiquitination has a therapeutic advantage over IKK inhibitors or proteasome inhibitors, we believe that our data would add a new insight into the development of a therapeutic approach against inflammatory diseases.

Second, our findings show that the inhibition of NF- κ B pathway by locally administered small-molecule inhibitor is potent as the treatment of allergic airway inflammation (Figs. 1 and 2). At present, the most efficient therapy of asthma is inhaled corticosteroids. However, there are a considerable number of patients who do not respond well to corticosteroid therapy [25]. Although precise mechanisms remain to be determined, several lines of evidence suggest the association of NF- κ B activation with the resistance to the corticosteroid therapy in severe asthma. For instance, it has been reported that NF- κ B activity and the production of pro-inflammatory cytokines from blood mononuclear cells are markedly enhanced in patients with severe asthma [26]. It has also been reported that local activation of NF- κ B in airway epithelial cells induces neutrophilic airway inflammation [27], which is a characteristics of severe asthma [28]. These findings suggest that local application of NF- κ B inhibitors might be an alternative therapeutic approach against severe asthma.

In conclusion, we show that the intranasal administration of I κ B ubiquitination inhibitor GS143 suppresses NF- κ B activation in the airways and attenuates subsequent allergic inflammation and mucus production in the airways. These results suggest that the inhibition of I κ B ubiquitination could be a therapeutic approach against asthma and that GS143 is one of candidates for the treatment of airway inflammation in asthmatic patients.

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