

Prevention of Asthma Exacerbation in a Mouse Model by Simultaneous Inhibition of NF- κ B and STAT6 Activation Using a Chimeric Decoy Strategy

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Transactivation of inflammatory and immune mediators in asthma is tightly regulated by nuclear factor κ B (NF- κ B) and signal transducer and activator of transcription 6 (STAT6). Therefore, we investigated the efficacy of simultaneous inhibition of NF- κ B and STAT6 using a chimeric decoy strategy to prevent asthma exacerbation. The effects of decoy oligodeoxynucleotides were evaluated using an ovalbumin-induced mouse asthma model. Ovalbumin-sensitized mice received intratracheal administration of decoy oligodeoxynucleotides 3 days before ovalbumin challenge. Fluorescent-dye-labeled decoy oligodeoxynucleotides could be detected in lymphocytes and macrophages in the lung, and activation of NF- κ B and STAT6 was inhibited by chimeric decoy oligodeoxynucleotide transfer. Consequently, treatment with chimeric or NF- κ B decoy oligodeoxynucleotides protected against methacholine-induced airway hyperresponsiveness, whereas the effect of chimeric decoy oligodeoxynucleotides was significantly greater than that of NF- κ B decoy oligodeoxynucleotides. Treatment with chimeric decoy oligodeoxynucleotides suppressed airway inflammation through inhibition of overexpression of interleukin-4 (IL-4), IL-5, and IL-13 and inflammatory infiltrates. Histamine levels in the lung were reduced via suppression of mast cell accumulation. A significant reduction in mucin secretion was observed due to suppression of MUC5AC gene expression. Interestingly, the inhibitory effects on IL-5, IL-13, and histamine secretion were achieved by transfer of chimeric decoy oligodeoxynucleotides only. This novel therapeutic approach could be useful to treat patients with various types of asthma.

INTRODUCTION

Allergic asthma is a common inflammatory disease of the airways, and its prevalence has markedly increased worldwide.¹ Therefore, the management of asthma is considered to be an important aspect of clinical practice. The pathologic condition of asthma is characterized by chronic inflammation, excess mucus production, and airway hyperresponsiveness (AHR).^{2,3} Glucocorticoids and/or bronchodilators are commonly used to treat and prevent the symptoms of asthma. However, long-term administration of these medicines often leads to adverse effects, especially in children, and some patients with asthma

are resistant to this conventional therapy.⁴ Although several biological drugs, such as anti-immunoglobulin E (anti-IgE) antibody and anti-interleukin-5 (anti-IL-5) antibody, have been explored and applied in clinical settings, an effective treatment option for several forms of allergic asthma is still an unmet medical need.⁵

In the process of asthma development, several types of cells, including both immune and structural cells, are activated according to environmental stimuli. The interactions among these cells induce variability in the pattern of pulmonary inflammation, including the migrated inflammatory cell profile. This phenomenon leads to the presence of subtypes or endotypes, which are thought to cause different responses to therapeutic agents.^{2,3,6} To treat this heterogeneous disease, we hypothesized that it is necessary to regulate a wide range of mediators associated with many aspects of asthma. Allergic proinflammatory and immune factors are tightly regulated by a transcriptional network system. Nuclear factor κ B (NF- κ B) and signal transducer and activator of transcription 6 (STAT6) play a central role in regulating transcription of these genes in various cell types. NF- κ B is well known to regulate numerous proinflammatory factors, including T helper type 2 (TH2) cytokines, and previous experimental studies of asthma using NF- κ B knockout mice have demonstrated that pulmonary inflammation and AHR were lacking in animals with deletion of the NF- κ B component.^{7,8} On the other hand, STAT6 is activated by IL-4, IL-5, and IL-13 and regulates the TH2 immune response in the pathogenesis of allergic asthma.⁹ In addition, inhibition of airway eosinophilia, mucus production, and AHR after allergen sensitization and challenge is observed in STAT6 knockout mice.^{10,11} Moreover, it

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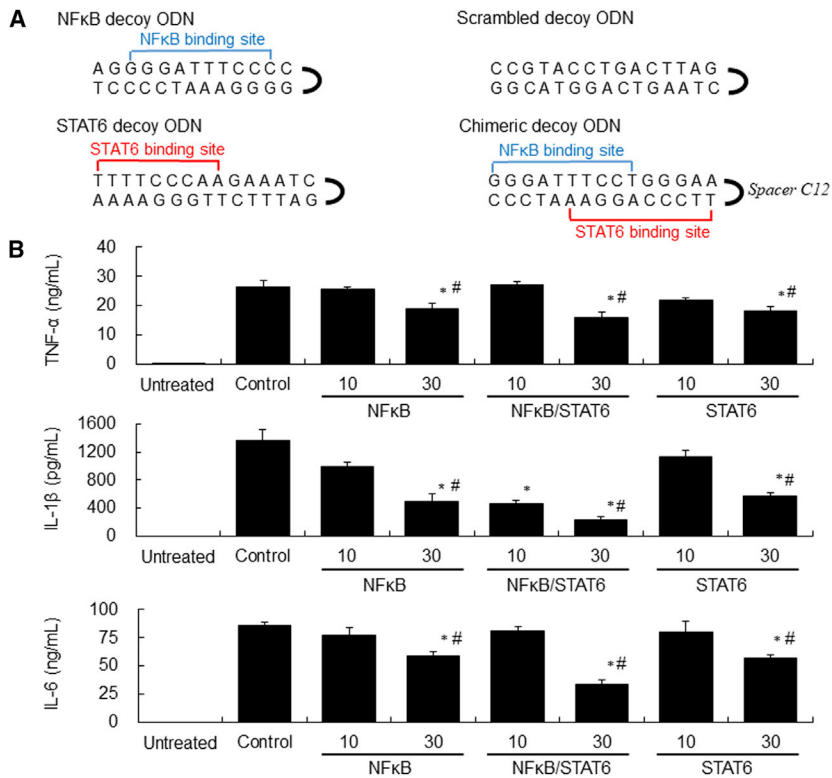


Figure 1. Construction of Decoy ODNs and Effects of Decoy ODNs on Inflammatory Cytokine Secretion *In Vitro*

(A) Sequences of NF-κB, STAT6, chimeric, and scrambled decoy ODNs. The chimeric decoy ODN contained overlapping binding sites for NF-κB and STAT6. (B) Effects of decoy ODNs on LPS-induced TNF-α, IL-1β, and IL-6 secretion in cell culture supernatants (n = 3 per group). 10, 10 nmol/L; 30, 30 nmol/L; control, untreated cells with stimulation only; NF-κB, treatment with NF-κB decoy ODN; NF-κB/STAT6, treatment with chimeric decoy ODN; ODN, oligodeoxynucleotide; STAT6, treatment with STAT6 decoy ODN; untreated, untreated cells without stimulation. Values are expressed as mean ± SEM. *p < 0.05 versus control, #p < 0.05 versus low-dose (10 nmol/L) decoy ODN.

transcription factors by intratracheal administration of a chimeric decoy ODN to prevent asthma exacerbation in a mouse model.

RESULTS

Inhibition of Cytokine Secretion by Chimeric Decoy ODN *In Vitro*

In this study, we employed a hairpin-type decoy ODN to increase stability, and a portion of the binding sites for NF-κB and STAT6 overlapped in the structure of the chimeric decoy ODN (Figure 1A).

The effects of inhibition of NF-κB and/or STAT6 on cytokine secretion were evaluated *in vitro* using a mouse macrophage cell line. Treatment with a high dose (30 nmol/L) of NF-κB, STAT6, or chimeric decoy ODN (NF-κB/STAT6) significantly inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF-α), IL-1β, and IL-6 secretion as compared to control. However, transfection of a low dose (10 nmol/L) of NF-κB or STAT6 decoy ODN did not suppress these cytokine elevations. In contrast, treatment with a low dose of chimeric decoy ODN significantly inhibited secretion of IL-1β in a dose-dependent manner (Figure 1B).

Distribution of 5-Carboxyfluorescein-Labeled Decoy ODN and Inhibition of Binding Activity of NFκB and STAT6

The effects of decoy ODNs were evaluated using an ovalbumin (OVA)-induced mouse asthma model. To confirm the successful transfer of decoy ODN in cells of the airway and lung by intratracheal administration, we examined the distribution of 5-carboxyfluorescein (FAM)-labeled decoy ODN in OVA-sensitized mice. FAM-labeled decoy ODN was intratracheally administered 3 days before OVA inhalation, and histological examination was performed at 24 hours after the last OVA challenge. Fluorescence was detected mainly in lymphocytes in the alveolar cavity and peribronchial region. Moreover, immunofluorescent staining revealed that fluorescence was also found in migrating macrophages (Figure 2A).

The effects of NF-κB and chimeric decoy ODN on the activation of transcription factors were evaluated by electrophoretic mobility shift

has been reported that NF-κB and STAT6 synergistically regulate an array of genes encoding cytokines and chemokines.⁹ Therefore, we focused on inhibition of NF-κB in combination with STAT6 using a chimeric decoy strategy to develop a novel therapeutic approach to treating asthma.

Decoy oligodeoxynucleotides (ODNs) are nucleic acid drugs regulating endogenous transcriptional activity.¹² They are synthetic double-stranded ODNs containing the consensus sequence of the binding site of the target transcription factor. In the nucleus, decoy ODNs can bind to target transcription factors, resulting in inhibition of the binding of the transcription factor to the promoter or enhancer region. As a result, transcription of genes regulated by the target transcription factor is repressed or activated, leading to normalization of the aberrant gene expression profile associated with disease progression.¹³ Based on this strategy, we developed a chimeric decoy ODN to inhibit multiple transcription factors. Chimeric decoy ODNs contain consensus sequences of multiple transcription factors in their structure, resulting in simultaneous inhibition of activation of target transcription factors. We have previously confirmed efficacy of the chimeric decoy strategy in several experimental studies.^{14,15} In addition, we developed a hairpin-type decoy ODN to increase resistance against endonuclease for intratracheal administration, because a major limitation of the usage of decoy ODNs *in vivo* is easy degradation by several nucleases.¹⁶

In this study, we employed a novel chimeric decoy ODN against NF-κB and STAT6 and examined the efficacy of inhibition of these

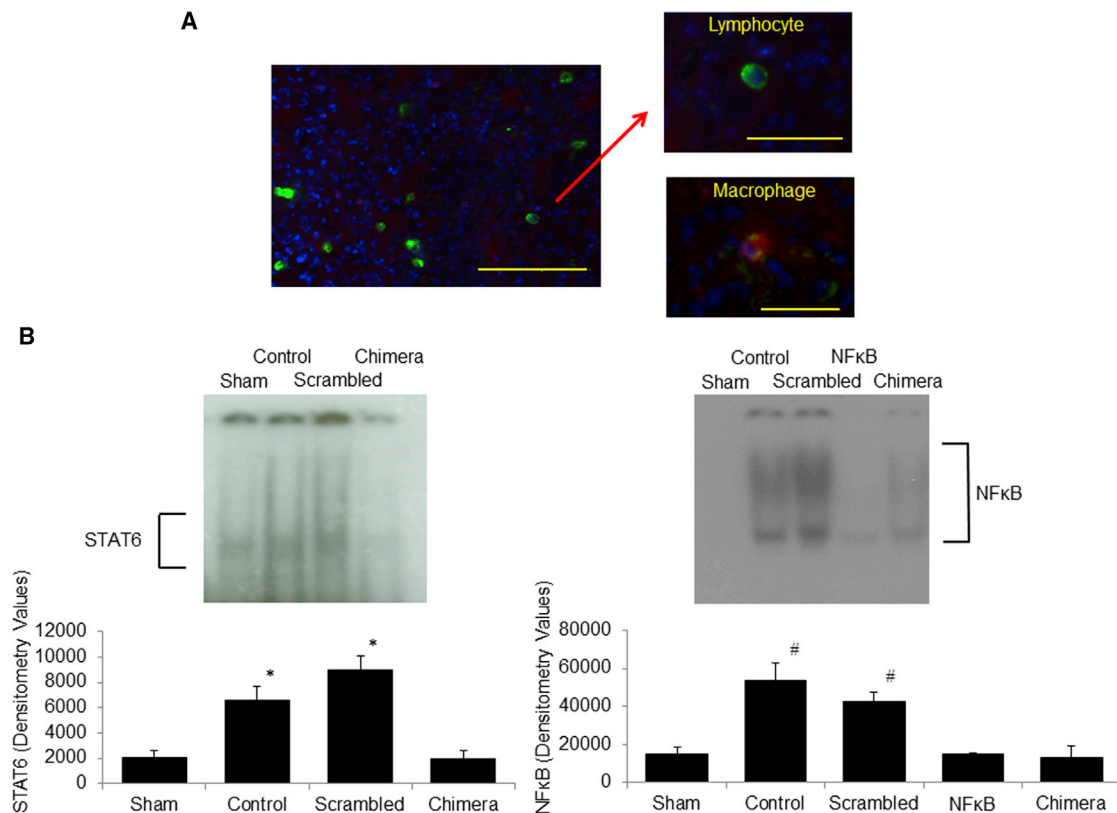


Figure 2. Distribution of FAM-Labeled Decoy ODNs and Inhibitory Effects of Decoy ODNs on the Binding Activity of NF-κB and STAT6

(A) Typical photograph of fluorescence in the lung. Fluorescence was detected mainly in lymphocytes at 6 days after intratracheal administration. Immunofluorescent staining revealed that FAM fluorescence was also found in migrating macrophages (red). (B) Representative results of electrophoretic mobility shift assay for NF-κB- and STAT6-binding sites and statistical analysis ($n = 4$ per group). Chimera, asthma model treated with chimeric decoy ODN; control, asthma model treated with saline; scrambled, asthma model treated with scrambled decoy ODN; NF-κB, asthma model treated with NF-κB decoy ODN; sham, sham-treated mice. Values are expressed as mean \pm SEM. Scale bars represent 100 μ m (A, left) and 50 μ m (A, right). * $p < 0.05$ versus sham and chimera; # $p < 0.05$ versus sham, NF-κB, and chimera.

assay (EMSA). The activity of both NF-κB and STAT6 was markedly increased in nuclear extracts of the lung in OVA-treated mice treated with saline (control) or scrambled decoy ODN. In contrast, transfection of chimeric decoy ODN significantly inhibited both NF-κB and STAT6 activation, and single transfection of NF-κB decoy ODN also inhibited the activation of NF-κB (Figure 2B). Although a portion of the binding sites for NF-κB and STAT6 overlapped, chimeric decoy ODN could effectively block the activation of both transcription factors *in vivo*.

Prevention of AHR by NF-κB and Chimeric Decoy ODNs

Because AHR is an important therapeutic target in the treatment of asthma, we investigated the effect of NF-κB and chimeric decoy ODNs on AHR aggravation in response to increasing doses of methacholine (MCh) in OVA-treated mice. After inhalation of MCh, airway resistance was markedly aggravated in mice treated with saline (control group) or scrambled decoy ODN in a dose-dependent manner, and there was no significant difference in response to MCh between these two groups. In contrast, treatment with NF-κB or chimeric decoy ODN significantly inhibited the increase in airway

resistance. Importantly, simultaneous inhibition of NF-κB and STAT6 completely normalized the response to MCh, and the effect of chimeric decoy ODN was significantly greater than that of single transfection of NF-κB decoy ODN (Figure 3).

Inhibition of Inflammation, Mucus Production, and Histamine Secretion by Chimeric Decoy ODN

To clarify the therapeutic effects of chimeric decoy ODN, we investigated the molecular mechanisms underlying prevention of asthma exacerbation. First, we evaluated the anti-inflammatory effects of chimeric decoy ODN. Although total cell number in bronchoalveolar lavage fluid (BALF) was increased in all groups after OVA sensitization and challenge, treatment with NF-κB or chimeric decoy ODN significantly suppressed the increase in cell number as compared to control or scrambled decoy ODN treatment. In addition, a significant reduction in the number of both eosinophils and neutrophils was also observed in mice treated with NF-κB or chimeric decoy ODN. Interestingly, the inhibitory effect of chimeric decoy ODN on pulmonary eosinophilia was significantly greater than that of NF-κB decoy ODN. In contrast, treatment with

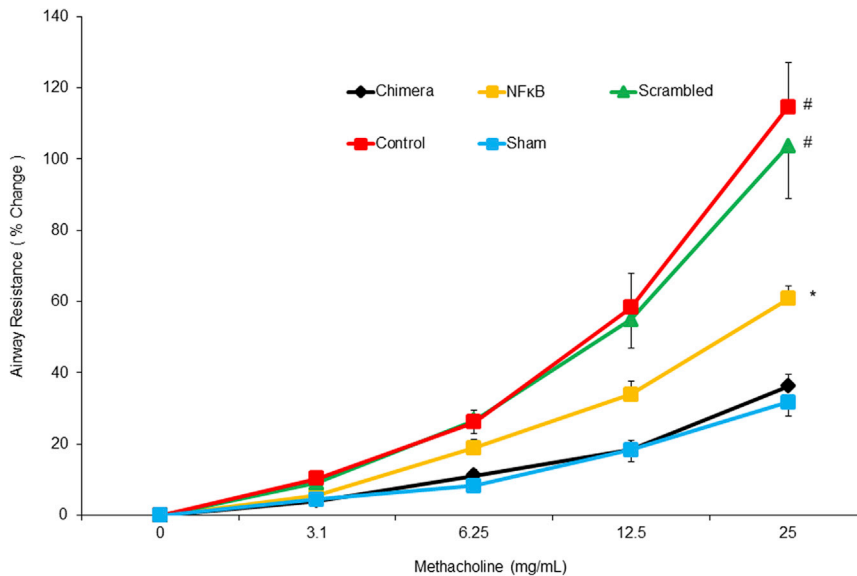


Figure 3. Effects of NF- κ B and Chimeric Decoy ODNs on AHR

Treatment with NF- κ B and chimeric decoy ODNs significantly inhibited aggravation of AHR. Airway resistance was determined relative to the baseline value in each mouse. Chimera, asthma model treated with chimeric decoy ODN (n = 9); control, asthma model treated with saline (n = 10); NF- κ B, asthma model treated with NF- κ B decoy ODN (n = 10); sham, sham-treated mice (n = 7); scrambled, asthma model treated with scrambled decoy ODN (n = 8). Values are expressed as mean \pm SEM. *p < 0.05 versus sham and chimera; #p < 0.05 versus sham, NF- κ B, and chimera.

NF- κ B or chimeric decoy ODN did not inhibit the increase in the number of lymphocytes (Figure 4).

In histological analysis, H&E staining demonstrated that the inflammatory infiltrate was markedly increased in the peribronchial and perivascular regions in control or scrambled decoy ODN-transferred mice. Unexpectedly, the accumulation of mononuclear cells was not inhibited by treatment with NF- κ B or chimeric decoy ODN (Figure 5A). However, immunohistochemical staining demonstrated that treatment with NF- κ B or chimeric decoy ODN significantly inhibited macrophage accumulation in the peribronchial region (Figure 5B).

Migrating inflammatory cells can secrete several proinflammatory mediators, and TH2 cytokines play an important role in the pathogenesis of asthma. Expression of IL-4, IL-5, and IL-13 in BALF was markedly increased in mice treated with saline or scrambled decoy ODN. However, treatment with chimeric decoy ODN significantly inhibited upregulation of these cytokines. In contrast, NF- κ B decoy ODN transfer inhibited IL-4 secretion only (Figure 6).

Next, the effect of decoy ODN on mucus production was investigated. Periodic acid-Schiff (PAS) staining demonstrated that mucus production in the lung was markedly increased in mice treated with saline or scrambled decoy ODN. However, treatment with NF- κ B or chimeric decoy ODN significantly inhibited mucus hypersecretion (Figures 7A and 7B). Although MUC5AC is the dominant mucin gene in the pathogenesis of asthma, treatment with NF- κ B or chimeric decoy ODN significantly inhibited upregulation of MUC5AC gene expression as compared to control or scrambled decoy ODN transfer (Figure 7C).

Finally, mast cell-related immune response was investigated in this model. The serum IgE level was increased in all groups after OVA

sensitization and challenge. Unexpectedly, treatment with NF- κ B or chimeric decoy ODN did not alter IgE production (Figure 8A). In contrast, histological analysis demonstrated that the number of IgE receptor (Fc ϵ RI)-positive cells was significantly decreased in lung tissues after NF- κ B or chimeric decoy ODN transfer. Interestingly, this inhibitory effect of chimeric decoy ODN was significantly greater than that of NF- κ B decoy ODN (Figure 8B). As a result, treatment with chimeric decoy ODN, but not NF- κ B decoy ODN alone, significantly decreased histamine levels in lung tissues (Figure 8C).

DISCUSSION

Simultaneous inhibition of both NF- κ B and STAT6 activity in the lung by intratracheal administration of chimeric decoy ODN induced a potent therapeutic effect on asthma exacerbation through inhibition of airway inflammation and the mast cell-mediated response, resulting in almost complete prevention of AHR and mucus hypersecretion. The therapeutic effect of inhibition of NF- κ B and STAT6 was significantly greater than that of inhibition of NF- κ B only, while NF- κ B decoy ODN could also prevent asthma exacerbation.

The pathological process of exacerbation of asthma involves multiple intracellular signaling pathways in immune and structural cells in response to experimental stimuli. These pathways converge on activation of a specific transcription factor network. Previous studies demonstrated that several transcription factors, such as NF- κ B, AP-1, STAT, and C/EBP, were activated in the lung tissue in patients with asthma and regulated many effector molecules.^{17–19} However, the effect of an individual transcription factor on transactivation of target genes differs among distinct cell types and disease phenotypes. Therefore, cross-talk among activated transcription factors is thought to mediate gain or loss of function in transcriptional regulation.^{9,19,20} These findings suggest that inhibition of multiple transcription factors is necessary to obtain sufficient target gene regulation to treat asthma and that several forms of asthma can be treated by regulating the transcription factor network. In our preliminary study selecting target transcription factors, inhibition of NF- κ B in combination with STAT6 was most effective in suppressing LPS-induced cytokine

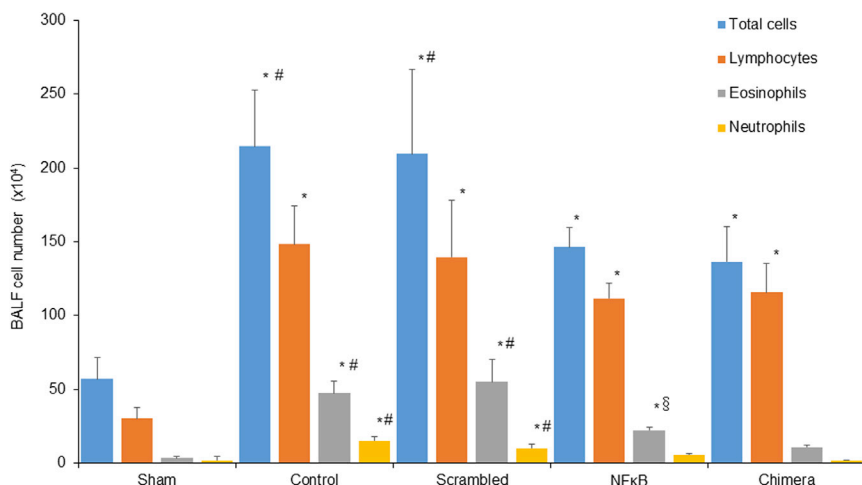


Figure 4. Inhibition of Inflammatory Cell Accumulation in the BALF by NF- κ B and Chimeric Decoy ODNs

Values are expressed as mean \pm SEM, n = 5 per group. *p < 0.05 versus sham; #p < 0.01 versus NF- κ B and chimera; §p < 0.05 versus chimera.

secretion in RAW 264.7 cells. In addition, recent studies reported that expression of several allergic mediators was under the control of both NF- κ B and STAT6.^{19,21} Therefore, NF- κ B and STAT6 could act synergistically to regulate the expression of many allergic mediators in asthma.

To inhibit activation of multiple transcription factors, we developed a chimeric decoy strategy and confirmed its efficacy in several inflammatory disease models, such as chimeric decoy ODN against NF- κ B and Ets for treating aortic aneurysm.^{14,15} Conventional chimeric decoy ODNs contain individual consensus sequences at a separate site in one decoy ODN. In the present study, a portion of the consensus sequences of NF- κ B and STAT6 (5 nt) overlapped in the structure of decoy ODN. Consequently, the length of ODN could be shortened, while activities of both target transcription factors were significantly inhibited in the *in vivo* study. Decoy ODNs with short sequences could increase the transfection efficiency and decrease the cost of production. In addition, sense and antisense strands of ODN were linked with a chemical spacer to increase the stability of ODN *in vivo*.¹⁶ As a result, despite a single administration, FAM-labeled decoy ODN could be detected in migrating inflammatory cells, and the therapeutic effect of NF- κ B or chimeric decoy ODN on asthma exacerbation was observed at least 6 days after intratracheal administration. Therefore, inhaled chimeric decoy ODN could act *in vivo* over a long period.

AHR is induced by interaction between several pathological conditions in the lung, and prevention of AHR is important for the management of asthma. In the present study, while aggravation of AHR in OVA-treated mice was significantly attenuated by NF- κ B decoy ODN, the effect was limited. A similar observation was reported in a previous experimental study of asthma.²² Therefore, inhibition of only NF- κ B activity might be insufficient for regulating allergic inflammation, because of the complex pathogenesis of asthma. In contrast, inhibition of both NF- κ B and STAT6 completely prevented AHR aggravation. IL-4, IL-5, and IL-13 are key mediators in the path-

ogenesis of asthma, including AHR aggravation, and inhibition of NF- κ B and STAT6 by inhaled chimeric decoy ODN effectively inhibited over-secretion of these cytokines. It has been reported that transcription of these cytokines is directly regulated by NF- κ B and STAT6 in various types of cells.^{3,5} In addition, although the main source of these cytokines is lymphocytes, decoy ODN could be transferred into these cells via intratracheal administration. Importantly, the inhibitory effect of chimeric decoy ODN on secretion of these cytokines was greater than that of NF- κ B decoy ODN, and a significant reduction in IL-5 and IL-13 levels in the BALF was achieved by treatment with chimeric decoy ODN only. These findings support a potent therapeutic effect of chimeric decoy ODNs.

AHR aggravation leads to bronchoconstriction, which is mainly induced by IgE-mediated release of constrictors from mast cells such as histamine. However, treatment with chimeric decoy ODNs could not affect IgE production, because transfection of decoy ODNs to cells by intratracheal administration was limited to the local site of the lung.²³ This distribution pattern might be a benefit to avoid systemic adverse events. In contrast, treatment with NF- κ B or chimeric decoy ODN inhibited accumulation of Fc ϵ RI-positive cells in the lung. Fc ϵ RI is usually found on mast cells and basophils, which are the main source of histamine secretion.²⁴ Recruitment of inflammatory cells, including mast cells, is mainly due to the expression of adhesion molecules and chemokines, and NF- κ B is well known to regulate the expression of these factors.²⁵ In addition, while stem cell factors also induce mast cell migration and activation, their gene expression is regulated by NF- κ B.²⁶ Therefore, mast cell accumulation would be suppressed by inhibition of NF- κ B activity. However, histamine levels in lung tissues were reduced by treatment with chimeric decoy ODN only. A recent study reported that STAT6 contributed to mast cell maturation, including granule formation, and the elevation of serum histamine levels after OVA sensitization and challenge was inhibited in STAT6 knockout mice.^{24,27} Therefore, activation of STAT6 is thought to contribute to mast cell-mediated responses in the effector phase, and treatment with chimeric decoy ODN might inhibit the effect of histamine on smooth muscle cell contraction. In addition, it has been reported that STAT6 and NF- κ B regulate IL-13- and TNF- α -induced AHR through enhanced expression of RhoA, which directly contracts smooth muscle cells.^{21,28} These findings suggest that chimeric decoy ODN could inhibit aggravated bronchoconstriction through direct and indirect effects.

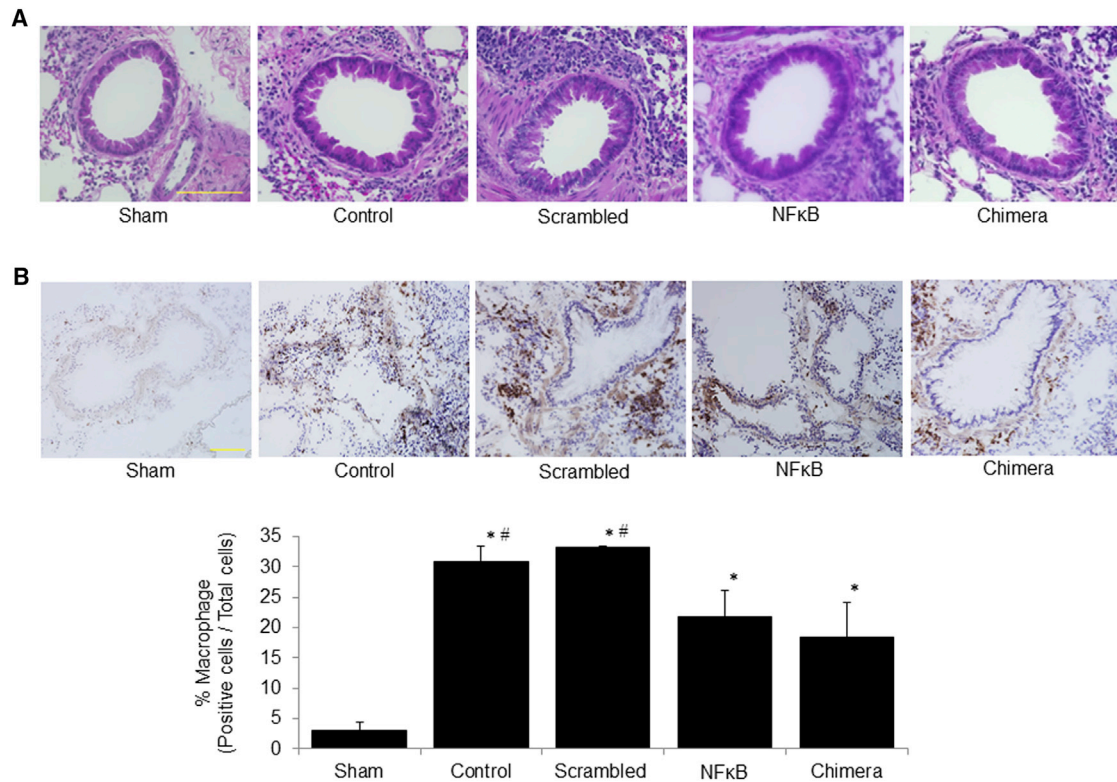


Figure 5. Inhibition of Inflammatory Infiltrates in the Lung by NF- κ B and Chimeric Decoy ODNs

(A) Representative histological sections of the lung stained with H&E after OVA challenge. (B) Typical example of immunohistochemical staining of macrophages (F4/80-positive cells) and percentage of positively stained cells in the lung ($n = 5$ per group). Values are expressed as mean \pm SEM. * $p < 0.01$ versus sham; # $p < 0.05$ versus NF- κ B and chimera. Scale bars, 100 μ m.

In addition to mast cell accumulation, various types of inflammatory cells migrate into the airway and lung tissue in the process of asthma exacerbation. While the migrating cell profile is different in each phenotype of asthma, treatment with chimeric decoy ODN could suppress recruitment of several types of inflammatory cells. Especially, eosinophils in the BALF were significantly decreased by NF- κ B or chimeric decoy ODN, and the effect of chimeric decoy ODN was significantly greater than that of NF- κ B decoy ODN. Pulmonary eosinophilia is correlated with the severity of asthma, and trafficking of eosinophils is mainly mediated by IL-5.^{2,3,5} Although both STAT6 and NF- κ B are well known to regulate the expression of IL-5, treatment with NF- κ B decoy ODN could not reduce IL-5 levels in the BALF in the present model. Therefore, the effect of NF- κ B decoy ODN on pulmonary eosinophilia might be mainly due to inhibition of adhesion molecule and chemokine expression. Accumulation of neutrophils and macrophages in the airways was also inhibited by treatment with chimeric decoy ODN. Although the precise role of these inflammatory cells in the pathogenesis of asthma is not clear, an increase in the number of these cells is a common feature in the lungs of patients with asthma.^{29–31} In addition, migrating neutrophils and macrophages are associated with pathogenesis in several phenotypes of asthma, such as neutrophilic asthma

and steroid resistance.^{31,32} These inflammatory cells secrete various inflammatory mediators, which leads to amplification of inflammatory responses and persistent inflammation in the airway via a consisting positive feedback loop to stimulate a transcription factor network. Indeed, treatment with chimeric decoy ODN inhibited the secretion of major inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in a dose-dependent manner in an *in vitro* study. These findings suggest that treatment with chimeric decoy ODN could be useful for treating several phenotypes of asthma. In contrast, lymphocytes are recruited into the airway from regional lymph nodes after exposure to allergen, and trafficking of lymphocytes is controlled by interaction between chemokines and their receptors on the surface of lymphocytes.^{33,34} In the initial phase of asthma exacerbation, the expression of these factors is induced by several discrete signaling pathways, including a STAT6-independent pathway.³⁴ Therefore, treatment with NF- κ B or chimeric decoy ODN could not inhibit the accumulation of lymphocytes in the airway and lung. However, transfection of decoy ODN could modulate the function of migrating lymphocytes in the airway.

Increased sputum is one of the main symptoms in asthma, and treatment with chimeric decoy ODN suppressed excess mucus production

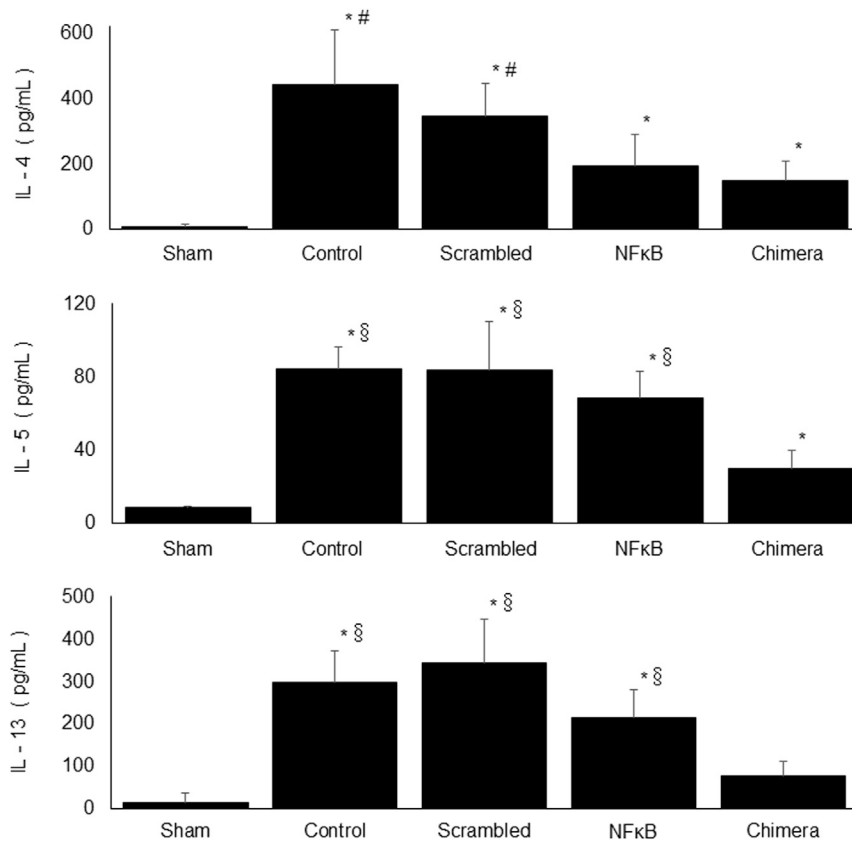


Figure 6. Inhibitory Effects of NF- κ B and Chimeric Decoy ODNs on Excess TH2 Cytokine Secretion in the BALF

Concentrations of IL-4, IL-5, and IL-13 in the BALF were measured by ELISA ($n = 5$ per group). Values are expressed as mean \pm SEM. * $p < 0.05$ versus sham; # $p < 0.05$ versus NF- κ B and chimera; § $p < 0.05$ versus chimera.

accompanied by inhibition of MUC5AC gene expression. Mucin 5AC is the main component of mucin and significantly contributes to asthma exacerbation. Previous studies demonstrated that NF- κ B and STAT6 regulate transactivation of the MUC5AC gene in goblet cells.^{35,36} In addition, although TH2 cytokines (such as IL-13) and histamine stimulate MUC5AC gene expression, these mediators were reduced by chimeric decoy ODN treatment.

In conclusion, although local inhibition of NF- κ B in the lung significantly inhibited asthma exacerbation, combined blockade of NF- κ B and STAT6 led to more potent therapeutic efficacy for treating asthma. The therapeutic effects were due to inhibition of a wide variety of mediators related to many aspects of asthma. Inhalation therapy of chimeric decoy ODN might be useful for treating patients with asthma in the clinical setting.

MATERIALS AND METHODS

Synthesis of ODN and Selection of Target Sequences

We employed double-stranded phosphorothioate scrambled, NF- κ B, STAT6, and chimeric (NF- κ B/STAT6) decoy ODNs, whose sequences are shown in Figure 1A. The two strands of decoy ODN between the 3' end of the sense strand and 5' end of the antisense strand were ligated through a linker to prepare a hairpin-type decoy ODN. The 5' end of the antisense strand was attached to a linker (spacer C12-[CH2]12), and then the sense strand was synthesized from the

other end of the linker. The complementary strands were hybridized by standard annealing.

Cell Culture and Measurement of Cytokine Secretion

The mouse macrophage cell line RAW 264.7 (RIKEN BioResource Center, Japan) was cultured in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO₂ incubator in a standard fashion. Cells were seeded onto 24-well tissue culture plates at a density of 3×10^4 cells per well and incubated for 24 hours. After incubation, cells were transfected with decoy ODN (10 and 30 nmol/L) combined with FuGENE HD Transfection Reagent (Promega). After 24 hours, cells were stimulated with LPS (1 μ g/mL). Concentrations of IL-1 β , IL-6, and TNF- α were measured in samples of cell culture supernatant using enzyme-linked immunosorbent assay kits (R&D Systems) after 24 hours of LPS stimulation ($n = 3$ per group).

Induction of OVA-Induced Allergic Asthma and Treatment with Decoy ODN

Female C57BL/6J mice (10–12 weeks of age; CLEA Japan, Japan) were sensitized on days 0 and 14 by intraperitoneal injection of 20 μ g OVA (grade V; Sigma-Aldrich, MO) dissolved in 150 μ L PBS with 2 mg (50 μ L) Imject Alum (Pierce Biotechnology, IL). On days 21, 22, and 23, the mice were challenged with an aerosol of 1% OVA in saline for 20 min using an ultrasonic nebulizer (NE-U07; Omron, Japan). Nonsensitized mice received an intraperitoneal injection of Imject Alum only with 150 μ L PBS and were challenged with saline using the same procedure employed for sham-treated mice.

To assess the effects of inhibition of NF- κ B and STAT6 on pulmonary allergic reaction, scrambled, NF- κ B, or chimeric decoy ODN (20 nmol in 25 μ L saline per mouse) was intratracheally administered using a MicroSprayer Aerosolizer (model 1A-1C; Penn-Century, PA), which can aerosolize a medicine-containing solution, on day 18 (3 days before OVA inhalation) to OVA-sensitized mice. Control and sham-treated mice were given saline (25 μ L) via intratracheal instillation. For administration of saline or decoy ODN, mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). Mice were randomly divided into five groups: sham

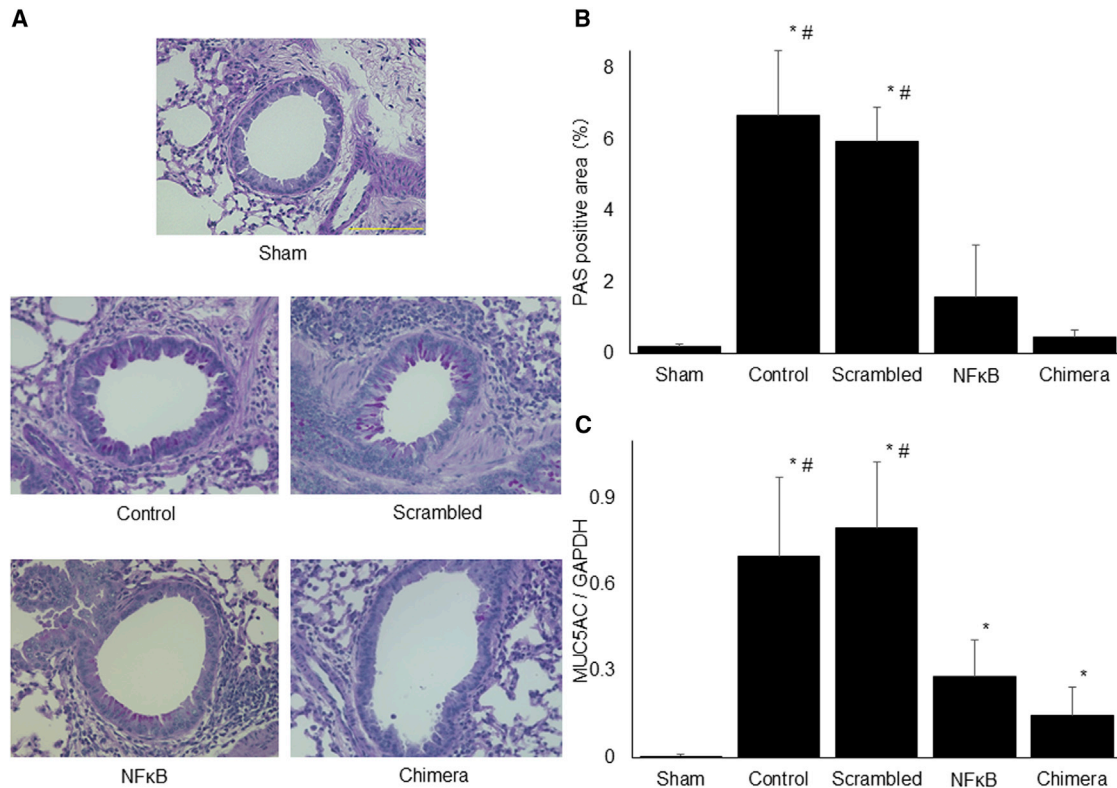


Figure 7. Prevention of Excess Mucus Production by NF- κ B and Chimeric Decoy ODNs

(A) Representative histological sections of lung stained with PAS after OVA challenge. (B) Percentage of PAS-positive area in the total epithelial cell area ($n = 5$ per group). (C) Real-time RT-PCR analysis of MUC5AC ($n = 5$ per group). Values are expressed as mean \pm SEM. * $p < 0.01$ versus sham; # $p < 0.01$ versus NF κ B and chimera. Scale bar, 100 μ m (A).

($n = 7$), treatment with saline (control; $n = 10$), scrambled decoy ODN ($n = 8$), NF- κ B decoy ODN ($n = 10$), and chimeric decoy ODN ($n = 9$).

Intratracheal administration of FAM-labeled chimeric decoy ODN was also performed in OVA-sensitized mice 3 days before OVA inhalation, and lung tissues were obtained at 24 hr after three daily OVA challenges. Frozen cross sections (5 μ m) of lung tissues were examined using a fluorescence microscope. The sections were also stained with rat monoclonal antibodies against F4/80 (1:500; Bio-Rad), and protein reacting with primary antibodies was visualized with fluorescence-conjugated anti-rat IgG antibodies (Alexa Fluor 568) to identify macrophages.

The experimental protocol was approved by the local Institutional Animal Care and Use Committee, and this study was performed under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Osaka University Medical School and the Japanese Government Animal Protection and Management Law (no. 105).

Measurement of AHR

24 hr after the last OVA challenge (day 24), AHR in response to MCh was measured in anesthetized mice. Under general anesthesia, mice

were tracheotomized with a cannula and mechanically ventilated (150 breaths/min, tidal volume 200 μ L), and the neuromuscular reaction was blocked by intramuscular injection of pancuronium bromide (0.1 mg/kg). Then, invasive airway resistance and compliance were measured using a Resistance and Compliance System (Buxco Electronics). After baseline reading, 10 μ L PBS and increasing concentrations of MCh (3.125, 6.25, 12.5, and 25 mg/mL) were nebulized into the tracheal tube for 30 s, followed by 270 s of incubation. Airway resistance was determined relative to the baseline value in each mouse.

Bronchoalveolar Lavage Analysis

BALF in each mouse was obtained immediately after AHR measurement. The airway lumen was washed three times with 0.4 mL ice-cold PBS. The total number of cells in the BALF was counted using a hemocytometer. Cells were stained with Diff-Quick solution, and differentials were performed based on morphology ($n = 5$ per group). Concentrations of IL-4 (R & D Systems), IL-5 (Thermo Fisher Scientific), and IL-13 (R&D Systems) in the BALF were determined by ELISA ($n = 5$ per group).

EMSA

Nuclear extracts were prepared from lung tissues after OVA challenge, and protein concentrations were carefully determined using

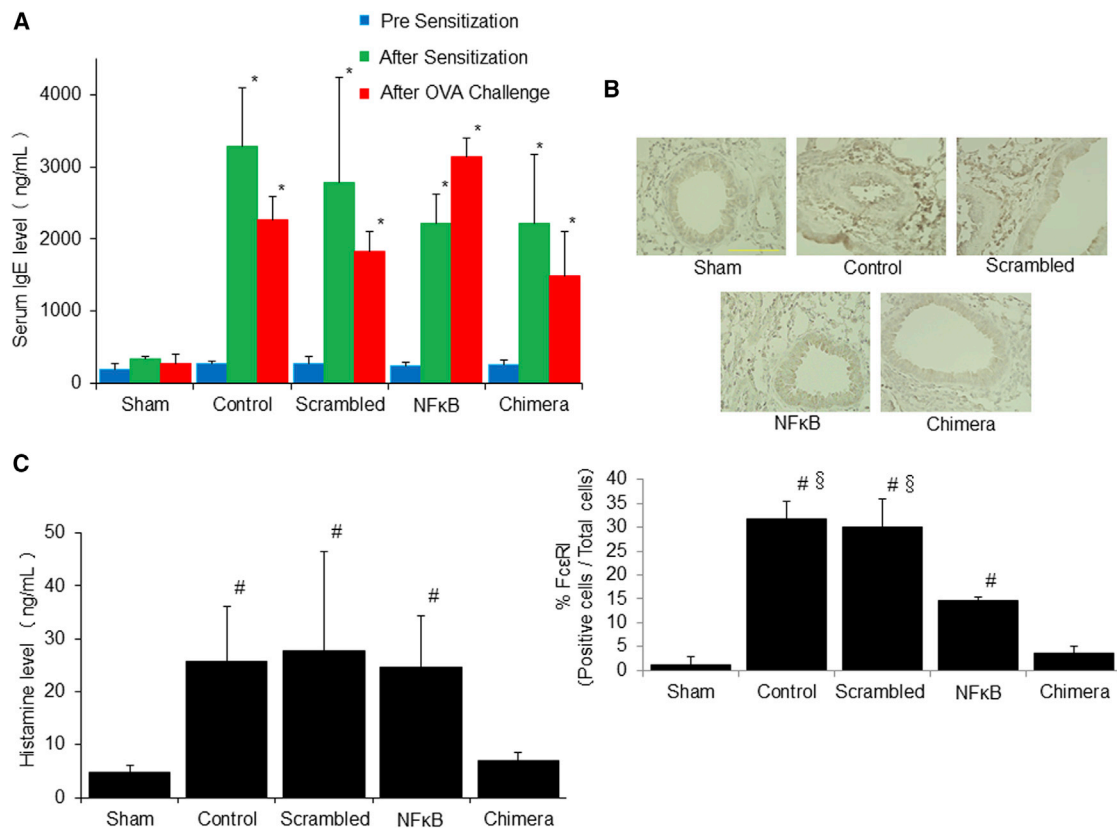


Figure 8. Inhibition of Histamine Production and Mast Cell Accumulation by Chimeric Decoy ODN

(A) Serum IgE level. There was no difference in total IgE level among all treated groups. (B) Typical example of immunohistochemical staining of FcεRI and percentage of positively stained cells in the lung. (C) Histamine levels in lung tissues. Values are expressed as mean \pm SEM, $n = 5$ per group. * $p < 0.01$ versus sham; # $p < 0.05$ versus sham and chimera; § $p < 0.05$ versus NF- κ B. Scale bar, 100 μ m (B).

the Bradford method.^{14,15} An EMSA was performed to analyze the expression of NF- κ B and STAT6 in nuclear extracts using a gel shift assay system ($n = 4$ per group; Promega, WI). ODN containing the NF- κ B-binding site (5'-CCTTGAAGGGATTTCCCTCC-3'; only the sense strand is shown) or the STAT6-binding site (5'-GTATTTCCAGAAAAGGAAC-3'; only the sense strand is shown) were labeled with [γ -³²P] ATP at the 3' end as a primer. Binding mixtures (10 μ L) including ³²P-labeled primers (10,000 cpm) and 1 μ g polydeoxyinosinic-deoxycytidic acid were incubated with 10 μ g nuclear extract for 20 min at room temperature and then loaded onto 6% polyacrylamide gel. As a control, samples were incubated with an excess (100 \times) of nonlabeled ODN, which completely abolished binding. The gels were subjected to electrophoresis, dried, and analyzed by autoradiography.

ELISA for Determining Serum IgE and Tissue Histamine Levels

Blood samples were collected on day 0 (before sensitization), day 21 (before OVA challenge), and day 24 (24 hr after the last OVA challenge), and total IgE levels in serum were measured by ELISA ($n = 5$ per group; AKRIE-010, Shibayagi, Japan). In addition, total protein was extracted from the homogenized lung tissue after OVA

challenge, and the protein concentration was carefully determined using the Bradford method. Histamine levels were measured in protein extracts (50 μ g, $n = 5$ per group) by ELISA (EA31, Oxford Biomedical Research, MI).

Histological and Immunohistochemical Studies

Animals were sacrificed on day 24. The right lower lobe of the lung was carefully dissected and processed for routine paraffin embedding. Lung tissue cross sections (6 μ m) were stained with both H&E and PAS in a standard manner. The PAS-positive area was measured in cross sections by quantitative morphometric analysis with a computerized sketching program. Results are expressed as a percentage of the total area of epithelial cells.

Immunohistochemical staining was performed using the immunoperoxidase avidin-biotin complex method (Vectastain Elite ABC kit). Immune complexes were localized using 0.05% 3,3'-diaminobenzidine, and slides were counterstained with hematoxylin. Paraffin sections were stained with rabbit FcεR1A antibodies (1:500; Bioworld Technology, MN) to analyze the expression of IgE receptors ($n = 5$ per group). Frozen cross sections (5 μ m) of the lung were also stained

with rat monoclonal antibodies against F4/80 (1:500; Bio-Rad) to analyze macrophage recruitment (n = 5 per group). For negative control experiments, nonimmune IgG was applied in place of the primary antibody. Positively stained cells and total cells were counted manually, and statistical analysis was performed.

Real-Time RT-PCR

Total RNA was extracted from the lung after OVA challenge with an RNeasy Mini Kit (n = 5 per group; QIAGEN, MD). cDNA was generated using a SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Quantitative real-time RT-PCR was performed using a TaqMan probe set for Muc5ac (Mm01276718-m1; Applied Biosystems, CA) and Realtime PCR Master Mix (Toyobo, Japan) with a Prism 7900HT Real-Time PCR System (Applied Biosystems, CA). Expression of Muc5ac was determined relative to the expression of GAPDH.

Statistical Analysis

Normality of distribution was tested using the Shapiro-Wilk test. Continuous variables with normal distribution were expressed as mean \pm SEM, and the Tukey-Kramer multiple range test was used for comparisons among multiple groups. $p < 0.05$ was considered significant.

AUTHOR CONTRIBUTIONS

Tetsuo Miyake, Takashi Miyake, M.S., H.N., and T.N. performed the experiments. T. Miyake and R.M. designed the experiments and wrote the paper.

CONFLICTS OF INTEREST

R.M. holds stock in AnGes.

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REFERENCES

- Eder, W., Ege, M.J., and von Mutius, E. (2006). The asthma epidemic. *N. Engl. J. Med.* 355, 2226–2235.
- Busse, W.W., and Lemanske, R.F., Jr. (2001). Asthma. *N. Engl. J. Med.* 344, 350–362.
- Cohn, L., Elias, J.A., and Chupp, G.L. (2004). Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.* 22, 789–815.
- Bush, A., and Saglani, S. (2010). Management of severe asthma in children. *Lancet* 376, 814–825.
- Hansbro, P.M., Scott, G.V., Essilfie, A.T., Kim, R.Y., Starkey, M.R., Nguyen, D.H., Allen, P.D., Kaiko, G.E., Yang, M., Horvat, J.C., and Foster, P.S. (2013). Th2 cytokine antagonists: potential treatments for severe asthma. *Expert Opin. Investig. Drugs* 22, 49–69.
- Wenzel, S.E. (2006). Asthma: defining of the persistent adult phenotypes. *Lancet* 368, 804–813.
- Donovan, C.E., Mark, D.A., He, H.Z., Liou, H.C., Kobzik, L., Wang, Y., De Sanctis, G.T., Perkins, D.L., and Finn, P.W. (1999). NF-kappa B/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. *J. Immunol.* 163, 6827–6833.
- Yang, L., Cohn, L., Zhang, D.H., Homer, R., Ray, A., and Ray, P. (1998). Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med.* 188, 1739–1750.
- Goenka, S., and Kaplan, M.H. (2011). Transcriptional regulation by STAT6. *Immunol. Res.* 50, 87–96.
- Kuperman, D., Schofield, B., Wills-Karp, M., and Grusby, M.J. (1998). Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med.* 187, 939–948.
- Akimoto, T., Numata, F., Tamura, M., Takata, Y., Higashida, N., Takashi, T., Takeda, K., and Akira, S. (1998). Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT) 6-deficient mice. *J. Exp. Med.* 187, 1537–1542.
- Morishita, R., Higaki, J., Tomita, N., and Ogihara, T. (1998). Application of transcription factor “decoy” strategy as means of gene therapy and study of gene expression in cardiovascular disease. *Circ. Res.* 82, 1023–1028.
- Miyake, T., Nakagami, H., and Morishita, R. (2012). Nucleic acid drugs for preventing restenosis after coronary revascularization. *Curr. Top. Med. Chem.* 12, 1613–1620.
- Miyake, T., Aoki, M., and Morishita, R. (2008). Inhibition of anastomotic intimal hyperplasia using a chimeric decoy strategy against NFkappaB and E2F in a rabbit model. *Cardiovasc. Res.* 79, 706–714.
- Miyake, T., Aoki, M., Masaki, H., Kawasaki, T., Oishi, M., Kataoka, K., Ogihara, T., Kaneda, Y., and Morishita, R. (2007). Regression of abdominal aortic aneurysms by simultaneous inhibition of nuclear factor kappaB and ets in a rabbit model. *Circ. Res.* 101, 1175–1184.
- Sen, M., Thomas, S.M., Kim, S., Yeh, J.L., Ferris, R.L., Johnson, J.T., Duvvuri, U., Lee, J., Sahu, N., Joyce, S., et al. (2012). First-in-human trial of a STAT3 decoy oligonucleotide in head and neck tumors: implications for cancer therapy. *Cancer Discov.* 2, 694–705.
- Gagliardo, R., Chanez, P., Mathieu, M., Bruno, A., Costanzo, G., Gougat, C., Vachier, I., Bousquet, J., Bonsignore, G., and Vignola, A.M. (2003). Persistent activation of nuclear factor-kappaB signaling pathway in severe uncontrolled asthma. *Am. J. Respir. Crit. Care Med.* 168, 1190–1198.
- Mullings, R.E., Wilson, S.J., Puddicombe, S.M., Lordan, J.L., Bucchieri, F., Djukanović, R., Howarth, P.H., Harper, S., Holgate, S.T., and Davies, D.E. (2001). Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium. *J. Allergy Clin. Immunol.* 108, 832–838.
- Caramori, G., Casolari, P., and Adcock, I. (2013). Role of transcription factors in the pathogenesis of asthma and COPD. *Cell Commun. Adhes.* 20, 21–40.
- Hoesel, B., and Schmid, J.A. (2013). The complexity of NF- κ B signaling in inflammation and cancer. *Mol. Cancer* 12, 86.
- Goto, K., Chiba, Y., Matsusue, K., Hattori, Y., Maitani, Y., Sakai, H., Kimura, S., and Misawa, M. (2010). The proximal STAT6 and NF-kappaB sites are responsible for IL-13- and TNF-alpha-induced RhoA transcriptions in human bronchial smooth muscle cells. *Pharmacol. Res.* 61, 466–472.
- Desmet, C., Gosset, P., Pajak, B., Cataldo, D., Bentires-Alj, M., Lekeux, P., and Bureau, F. (2004). Selective blockade of NF-kappa B activity in airway immune cells inhibits the effector phase of experimental asthma. *J. Immunol.* 173, 5766–5775.
- Robinson, D.S. (2004). The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *J. Allergy Clin. Immunol.* 114, 58–65.
- Ryan, J.J., DeSimone, S., Klisch, G., Shelburne, C., McReynolds, L.J., Han, K., Kovacs, R., Mirmonsef, P., and Huff, T.F. (1998). IL-4 inhibits mouse mast cell Fc epsilonRI expression through a STAT6-dependent mechanism. *J. Immunol.* 161, 6915–6923.
- Edwards, M.R., Bartlett, N.W., Clarke, D., Birrell, M., Belvisi, M., and Johnston, S.L. (2009). Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol. Ther.* 121, 1–13.
- Da Silva, C.A., Heilbock, C., Kassel, O., and Frossard, N. (2003). Transcription of stem cell factor (SCF) is potentiated by glucocorticoids and interleukin-1beta through concerted regulation of a GRE-like and an NF-kappaB response element. *FASEB J.* 17, 2334–2336.

27. Han, N.R., Oh, H.A., Nam, S.Y., Moon, P.D., Kim, D.W., Kim, H.M., and Jeong, H.J. (2014). TSLP induces mast cell development and aggravates allergic reactions through the activation of MDM2 and STAT6. *J. Invest. Dermatol.* *134*, 2521–2530.
28. Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., and Donaldson, D.D. (1998). Interleukin-13: central mediator of allergic asthma. *Science* *282*, 2258–2261.
29. Barnes, P.J. (2008). Immunology of asthma and chronic obstructive pulmonary disease. *Nat. Rev. Immunol.* *8*, 183–192.
30. Schulman, E.S., Liu, M.C., Proud, D., MacGlashan, D.W., Jr., Lichtenstein, L.M., and Plaut, M. (1985). Human lung macrophages induce histamine release from basophils and mast cells. *Am. Rev. Respir. Dis.* *131*, 230–235.
31. Pappas, K., Papaioannou, A.I., Kostikas, K., and Tzanakis, N. (2013). The role of macrophages in obstructive airways disease: chronic obstructive pulmonary disease and asthma. *Cytokine* *64*, 613–625.
32. Ordoñez, C.L., Shaughnessy, T.E., Matthay, M.A., and Fahy, J.V. (2000). Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance. *Am. J. Respir. Crit. Care Med.* *161*, 1185–1190.
33. Harris, N.L., Watt, V., Ronchese, F., and Le Gros, G. (2002). Differential T cell function and fate in lymph node and nonlymphoid tissues. *J. Exp. Med.* *195*, 317–326.
34. Medoff, B.D., Thomas, S.Y., and Luster, A.D. (2008). T cell trafficking in allergic asthma: the ins and outs. *Annu. Rev. Immunol.* *26*, 205–232.
35. Fujisawa, T., Velichko, S., Thai, P., Hung, L.Y., Huang, F., and Wu, R. (2009). Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *J. Immunol.* *183*, 6236–6243.
36. Yu, H., Li, Q., Kolosov, V.P., Perelman, J.M., and Zhou, X. (2010). Interleukin-13 induces mucin 5AC production involving STAT6/SPDEF in human airway epithelial cells. *Cell Commun. Adhes.* *17*, 83–92.