Receptor-Interacting Protein 2 Gene Silencing Attenuates Allergic Airway Inflammation

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*J Immunol* 2013; 191:2691-2699; Prepublished online 5 August 2013; doi: 10.4049/jimmunol.1202416

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Persistent activation of NF-κB has been associated with the development of asthma. Receptor-interacting protein 2 (Rip2) is a transcriptional product of NF-κB activation. It is an adaptor protein with serine/threonine kinase activity and has been shown to positively regulate NF-κB activity. We investigated potential protective effects of Rip2 gene silencing using small interfering RNA (siRNA) in an OVA-induced mouse asthma model. Rip2 protein level was found to be upregulated in allergic airway inflammation. A potent and selective Rip2 siRNA given intratracheally knocked down Rip2 expression in OVA-challenged lungs and reduced OVA-induced increases in total and eosinophil counts, and IL-4, IL-5, IL-13, IL-1β, IL-33, and eosinax levels in bronchoalveolar lavage fluid. Rip2 silencing blocked OVA-induced inflammatory cell infiltration and mucus hypersecretion as observed in lung sections, and mRNA expression of ICAM-1, VCAM-1, E-selectin, RANTES, IL-17, IL-33, thymic stromal lymphopoietin, inducible NO synthase, and MUC5ac in lung tissues. In addition, elevation of serum OVA-specific IgE level in mouse asthma model was markedly suppressed by Rip2 siRNA, together with reduced IL-4, IL-5, and IL-13 production in lymph node cultures. Furthermore, Rip2 siRNA-treated mice produced significantly less airway hyperresponsiveness induced by methacholine. Mechanistically, Rip2 siRNA was found to enhance cytosolic level of IκBα and block p65 nuclear translocation and DNA-binding activity in lung tissues from OVA-challenged mice. Taken together, our findings clearly show that knockdown of Rip2 by gene silencing ameliorates experimental allergic airway inflammation, probably via interruption of NF-κB activity, confirming Rip2 a novel therapeutic target for the treatment of allergic asthma.


from OVA mouse asthma model (6). In mice deficient in NF-κB p50 subunit or IκBβ, reduced pulmonary eosinophilia, Th2 cytokine and eotaxin levels, and mucus production were observed in response to OVA aerosol challenge (7, 8).

Receptor-interacting protein 2 (Rip2), also known as CARD3, CARDICK, or RICK2, consists of an N-terminal serine/threonine kinase domain and a CARD domain for protein–protein interaction (9). Rip2 has been shown to mediate TCR signaling to NF-κB activation, cytokine production, and T cell proliferation, but the serine/threonine kinase activity is not required for NF-κB response (9–11). Rip2 is an inducible transcriptional product of NF-κB activation, and serves as a positive regulator of NF-κB pathway by binding to the IKK complex (12). In vitro study revealed that overexpression of Rip2 in HEK293T cells increases NF-κB activity and IL-1β production (13). In addition, an association study using linkage disequilibrium mapping has linked Rip2 gene to severe childhood asthma (14). The purpose of the current study was to investigate the role of Rip2 in allergic airway inflammation in an OVA mouse asthma model.

Small interfering RNA (siRNA) has been extensively used to knockdown specific protein targets in the lungs as a research tool and as a therapeutic modality. Upon entering the cells, the double-stranded siRNA is recruited to the RNA-induced silencing complex, leading to unwinding of the duplex into a passenger strand and a guide (antisense) strand, which recognizes the target mRNA. The sequence-specific binding results in a RNA-induced silencing complex–mediated cleavage and degradation of the target mRNA, and a resultant drop in target protein expression. siRNA can be directly instilled into the lungs noninvasively via intranasal or intratracheal administration in doses much lower than systemic delivery with reduced nonspecific systemic side effects (15, 16). In addition, the lung has a very large absorption surface area, and it is lined with surfactant, a cationic glycolipid at physiologic
pH, which serves as a vehicle to facilitate siRNA uptake into lung cells (17).

To our knowledge, we observed for the first time that lung Rip2 protein level was markedly elevated in allergic airway inflammation, and Rip2 siRNA potently knocked down Rip2 and suppressed OVA-induced inflammatory cell infiltration, mucus hypersecretion, cytokine and inflammatory mediator productions, and airway hyperresponsiveness (AHR). The anti-inflammatory actions of Rip2 siRNA are probably mediated by the interruption of OVA-induced NF-κB nuclear translocation and transactivation in the lungs. Our findings clearly indicate that Rip-2 is a novel therapeutic target for the treatment of allergic asthma.

Materials and Methods

In vitro characterization of Rip2 siRNA

A mouse macrophage cell line RAW264.7 and a mouse fibroblast cell line NIH/3T3 (American Type Culture Collection, Rockville, MD) were maintained in DMEM (Invitrogen, Carlsbad, CA). To study Rip2 gene silencing, cells were transfected with 100 nM Rip2 siRNA or negative siRNA control for 6 h at 37˚C in OptiMEM (Invitrogen) containing Lipofectamine 2000 (Invitrogen). ON-TARGETplus siRNA complementary to mouse Rip2 mRNA with the following sequences: S1, 5’-GCUCGACAGUGAAAGA-3’; S2, 5’-ACGAGAAGCCGAAAUAUUA-3’; and S3, 5’-CAAAUUCUCCCUCAGAAUAA-3’, and custom-made nontargeting control siRNA, 5’-UUCUCCGAACGUGUCACGU-3’, were purchased from Thermo Scientific (Waltham, MA). Transfected cells were allowed to recover for 18, 42,

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or 66 h in complete DMEM before they were analyzed for Rip2 mRNA and protein expression.

**Animals**

Female BALB/c mice 6–8 wk of age (Animal Resources Center, Canning Vale, Western Australia, Australia) were sensitized by i.p. injections of 20 μg OVA and 4 mg Al(OH)3 suspended in 0.1 ml saline on days 0 and 14. On days 22, 23, and 24, mice were challenged with 1% OVA aerosol for 30 min. Rip2 siRNA (1 and 5 nmol) or negative control siRNA in 30 μl PBS was given daily via intratracheal route on days 19–21, and 2 h before each OVA aerosol challenge on days 22–24. Animal experiments were performed according to the institutional guidelines for Animal Care and Use Committee of the National University of Singapore.

**Bronchoalveolar lavage fluid and serum analyses**

Mice were anesthetized 24 h after the last OVA challenge, and bronchoalveolar lavage (BAL) was performed, as described (18). BAL fluid total and differential cell counts and cytokine and chemokine levels were determined, as described (18). Blood was collected by cardiac puncture, and serum levels of total IgE and OVA-specific IgE were determined, as described (6).

**Lymph node cell cultures**

To determine the effects of Rip2 siRNA on OVA-specific immune responses in lymphocytes, thoracic lymph nodes were removed from the lungs and passed through cell strainers (BD Biosciences, San Jose, CA) to prepare a single-cell suspension. Cells were cultured at 2 × 10^6 cells/ml in complete RPMI 1640 medium supplemented with 10% heat-inactivated FCS for 72 h (18). Supernatants from parallel triplicate cultures were analyzed for cytokine levels by ELISA.

**Histologic analysis**

Lungs were fixed in 10% neutral formalin, paraffinized, cut into 5-μm sections, and stained with H&E for examining cell infiltration and with periodic acid–fluorescence Schiff stain for mucus production (18). Periodic acid–fluorescence Schiff stain allows visualization of mucus through covalent bonding of sulfited acriflavine to mucin glycoconjugates. Mucin granules emit red fluorescence when excited at 380–580 nm and observed at 600–650 nm using a confocal microscope (Leica TCS SP5; Leica Microsystems, Deerfield, IL). Noncovalent linkage of acriflavine to nucleic acid, nuclei, and cytoplasm results in green fluorescence when excited at 380–500 nm and observed at 450–475 nm. Quantitative analyses of cell infiltration and mucus production were performed blinded, as described (6, 17).

**Measurements of AHR**

Mice were anesthetized, and tracheotomy and intubation were performed (18). The trachea was intubated with a cannula that was connected to the pneumotach, ventilator, and nebulizer. Lung resistance (Rl) and dynamic compliance (Cdyn) in response to nebulized methacholine (0.5–8.0 mg/ml) were recorded using a whole-body plethysmography chamber and the FinePointe data acquisition and analysis software (Buxco, Wilmington, NC), as described (18). Results are expressed as a percentage of the respective basal values in response to PBS.

**Immunoblotting, NF-κB DNA binding, and mRNA expression**

To determine NF-κB nuclear translocation, lung cytosolic and nuclear extracts (30 μg/lane) were separated by 10% SDS-PAGE, and immunoblots were probed with anti-p65 mAb, anti-IκBα mAb (Cell Signaling, Beverly, MA), or Rip2 mAb (Abcam, Cambridge, U.K.), and anti-β-actin and anti-TATA binding protein (TBP; Abcam) Abs as internal controls. Band intensity was quantitated using ImageJ software (NIH), as described (18). Nuclear proteins were also analyzed for NF-κB DNA binding using the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA). Total mRNA was extracted using TRizol reagent (Invitrogen); cDNA was then reverse transcribed from 1 μg isolated RNA, PCR amplifications were performed using primers listed in Table I, and the PCR products were separated in a 2% agarose gel and visualized under UV light or analyzed by quantitative real-time PCR (ABI 7500 Cycler; Applied Biosystems, Carlsbad, CA). The mRNA expression levels were normalized to the level of the housekeeping gene β-actin.

**Statistical analysis**

Data are presented as means ± SEM. One-way ANOVA followed by Dunnett’s test was used to determine significant differences between treatment groups. Significant levels were set at p < 0.05.
Results

In vitro characterization of Rip2 siRNA

We have screened the gene-silencing effects of three Rip2 siRNA sequences (S1–S3) targeted at different sites of the coding region of mouse Rip2 mRNA, in both RAW264.7 and NIH/3T3 cell lines. S1, S2, and S3 markedly silenced Rip2 mRNA expression (Table I) in both cell lines 24 h after transfection by ~70%, as compared with the control siRNA (Fig. 1A). The three sequences produced equivalent knockdown of Rip2 protein expression by at least 80% in RAW264.7 cells and NIH/3T3 cells at 48 and 72 h, respectively (Fig. 1B). Among the three sequences, S2 consistently knocked down Rip2 with the least variability and was chosen as the lead siRNA for subsequent in vivo experiments.

Rip2 gene silencing in vivo

Daily intratracheal administration of 5 nmol S2 to naive BALB/c mice for consecutive 3 d was able to knock down Rip2 protein lung level for up to 72 h after the last siRNA dose (Fig. 2A). In OVA mouse asthma model, to our knowledge, we observed for the first time that Rip2 lung level was markedly elevated (Fig. 2B). To ensure lung Rip2 protein knockdown in asthma, we compared the gene-silencing capacities of 3 daily-dose regimen of S2 before starting OVA aerosol challenge and 6 daily-dose regimen of S2 (3 doses before OVA challenge plus 3 additional doses during OVA aerosol challenges). Only the 6 daily-dose regimen of S2 was able to downregulate Rip2 lung level in mouse asthma model (Fig. 2C). To confirm S2 specificity for Rip2 mRNA, a BLAST search was conducted and has revealed two closest complementary sequences to S2 siRNA, which are from the TLR3 and Zn finger HIT domain-containing protein 3 (Znhit-3), a thyroid receptor-interacting protein. S2 did not show any silencing effect on TLR3 and Znhit-3 gene expression in lung tissues from mouse asthma model (Fig. 2D).

Rip2 siRNA suppresses OVA-induced inflammatory cell recruitment and mucus production

OVA inhalation markedly increased total cell, eosinophil, and macrophage counts, and slightly, yet significantly increased lymphocyte and neutrophil counts, as compared with the saline aerosol control (Fig. 3A). Intratracheal S2 (1 and 5 nmol) drastically decreased the total cell and eosinophil counts in BAL fluid in a dose-dependent manner as compared with the control siRNA (Fig. 3A). In addition, S2 at 5 nmol significantly reduced BAL fluid neutrophil count (Fig. 3A). To further ensure specificity of Rip2 gene silencing, S1 siRNA was also tested in the OVA mouse asthma model. In a similar fashion, intratracheal S1 (5 nmol) markedly suppressed the total and eosinophil counts in BAL fluid as compared with control siRNA (Fig. 3B). OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchial and perivascular connective tissues as compared with saline challenge. S2 (5 nmol) significantly attenuated the eosinophil-rich leukocyte infiltration as compared with control siRNA (Fig. 3C). In contrast, OVA-challenged mice, but not saline-challenged mice, developed marked mucus hypersecretion in the bronchi. S2 (5 nmol) substantially reduced mucus hypersecretion as compared with control siRNA (Fig. 3D).

FIGURE 3. Effects of Rip2 gene silencing on OVA-induced inflammatory cell recruitment and mucus hypersecretion. (A) Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 h after the last saline aerosol (n = 6 mice) or OVA aerosol (n = 7 mice) challenge. S2 dose dependently reduced OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 h after the last OVA aerosol challenge (siRNA-negative control (Con), n = 7 mice; 1 nmol S2, n = 7 mice; 5 nmol S2, n = 8 mice). (B) Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 h after the last saline aerosol (n = 4 mice) or OVA aerosol (n = 4 mice) challenge. Intratracheal S1 (5 nmol) significantly suppressed OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 h after the last OVA aerosol challenge (Con, n = 4; 5 nmol S1, n = 4 mice). Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Histologic sections of lung tissue eosinophilia (C) (original magnification ×200) and mucus secretion (D) (original magnification ×1000) obtained 24 h after the last aerosol challenge with and without S2 treatment were evaluated. Quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed, as previously described (6). Scoring of inflammatory cells and mucus secretion was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from siRNA-negative control (Con), p < 0.05.
Rip2 siRNA reduces OVA-induced BAL fluid cytokines and serum IgE

OVA aerosol challenge produced a notable increase in IL-4, IL-5, IL-13, IL-1β, IL-33, and eotaxin levels in BAL fluid as compared with saline aerosol controls. Rip2 siRNA dose dependently suppressed IL-4, IL-5, IL-13, IL-1β, IL-33, and eotaxin as compared with control siRNA (Fig. 4A). To evaluate whether Rip2 gene silencing could modify an ongoing OVA-specific Th2 response in vivo, serum levels of total IgE and OVA-specific IgE were determined. Marked elevations in serum total IgE and OVA-specific IgE levels were observed in OVA-challenged mice as compared with saline-challenged mice. Rip2 gene silencing significantly suppressed OVA-specific IgE levels in a dose-dependent manner, and, to a lesser extent, the serum level of total IgE (Fig. 4B).

Rip2 siRNA suppresses OVA-specific lymphocyte responses in vitro

From the above observations, it seemed likely that the polarity of OVA-specific responses had been modified by Rip2 gene silencing. We therefore examined the OVA-specific immune responses in thoracic lymph node cultures to assess whether Rip2 siRNA treatment directly influenced lymphocyte function. OVA-induced production of IL-4, IL-5, and IL-13 from lymph node cells was significantly reduced in S2-treated mice as compared with control siRNA-treated mice (Fig. 5). This immune modulation by Rip2 gene silencing in vivo was Ag specific as Con A-induced production of IL-4, IL-5, IL-13, and IFN-γ in parallel cultures was not affected (data not shown).

Rip2 siRNA suppresses OVA-induced inflammatory gene expression in lungs

OVA aerosol challenge markedly upregulated mRNA levels of lung adhesion molecules (ICAM-1, VCAM-1, and E-selectin), chemokine (RANTES), proinflammatory cytokines (thymic stromal lymphopoietin [TSLP], IL-17, IL-33, and TNF-α), and inflammatory mediators (inducible NO synthase [iNOS] and Muc5ac). Intratracheal S2 (5 nmol) demonstrated strong suppression of all these proinflammatory mediators in the allergic airways as compared with control siRNA treatment (Fig. 6).

Rip2 siRNA reduces OVA-induced AHR

To investigate the effect of Rip2 gene silencing on AHR, we measured both Rl and Cdyn in mechanically ventilated mice. Rl is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. OVA-challenged mice developed AHR to increasing concentrations of methacholine, which is typically reflected by high Rl and low Cdyn. Intratracheal S2 (5 nmol) significantly reduced Rl and restored Cdyn in OVA-challenged mice in response to methacholine, suggesting that immune-mediated airway pathology in vivo was modified (Fig. 7).

Rip2 gene silencing disrupts NF-κB signaling pathway

To verify that Rip2 is a positive regulator of NF-κB pathway, we examined Rip2 gene silencing on IkBα protein level and NF-κB p65 subunit nuclear translocation and transactivation in lung tissues obtained 24 h after the last OVA or saline aerosol challenge. OVA challenge markedly decreased the cytosolic level of IkBα, and promoted p65 nuclear translocation, as evidenced by a drastic drop in cytosolic p65 level and a corresponding surge in nuclear p65 level in lung tissues as compared with those from saline aerosol control (Fig. 8A). In addition, OVA challenge substantially enhanced nuclear p65 DNA-binding activity (Fig. 8B). Intratracheal S2 (5 nmol) significantly (p < 0.05) maintained cytosolic IkBα level, retained p65 in the cytosol, and halted p65 nuclear translocation and DNA-binding activity in OVA-challenged lungs as compared with control siRNA (Fig. 8).

**FIGURE 4.** Effects of Rip2 gene silencing on OVA-induced BAL fluid cytokine and chemokine levels and serum IgE levels. (A) BAL fluids were collected 24 h after the last aerosol challenge. Levels of IL-4, IL-5, IL-13, IL-1β, IL-33, and eotaxin were analyzed using ELISA (n = 6–9 mice per group). (B) Mouse serum was collected 24 h after the last aerosol challenge. The levels of total IgE and OVA-specific IgE were analyzed using ELISA (n = 5–9 mice per group). Values shown are the mean ± SEM. *Significant difference from siRNA-negative control (Con), p < 0.05.

**Discussion**

Persistent NF-κB activation has been observed in allergic airway inflammation (1, 2). Various strategies targeted at the NF-κB signaling pathway, such as NF-κB–specific decoy oligonucleotide (19), p65-specific antisense oligonucleotide (20), and IKKβ-selective small molecule inhibitor (21), have demonstrated beneficial effects in experimental asthma models. Rip2 is not only a transcriptional product of NF-κB, but also a positive regulator of NF-κB activity (12). In this study, to our knowledge, we observed for the first time that lung Rip2 protein level was markedly elevated in experimental asthma, which was accompanied by increased NF-κB nuclear translocation and DNA binding. It has been reported that overexpression of Rip2 in HEK293T cells promoted NF-κB activation (13). Stimulated T cells from Rip2 knockout mice exhibited diminished NF-κB activity (10). Likewise, Rip2-deficient macrophages were defective in NF-κB activation and cytokine production in response to LPS stimulation.
In line with this, suppression of Rip2 expression using Rip2 siRNA resulted in abrogation of NF-κB activation (23) and reduction of TSLP and IL-1β expression in mast cells (24, 25).

We have characterized a Rip2-specific siRNA (S2) capable of knocking down Rip2 mRNA and protein levels by at least 70–80% in two cell lines, without nonspecific gene-silencing effect on TLR3 and Znhit-3, which encode closely resembling mRNA sequence complementary to S2 siRNA. It has been demonstrated that Rip2 serves as a scaffolding structure directly interacting with IKKγ (NF-κB essential modifier), rendering the IKK complex functional and leading to NF-κB activation (26, 27). In addition, Rip2-mediated NF-κB activation does not require its serine/threonine kinase activity (9–11). In this study, intratracheal administration of Rip2 siRNA to experimental asthma mice resulted in a marked...
lung Rip2 knockdown, and mitigation of OVA-induced inflammatory cell infiltration; airway mucus hypersecretion; cytokine, chemokine, and proinflammatory mediator productions; and AHR. These observations were also accompanied by an inhibition of p65 nuclear translocation and κB DNA-binding activity in OVA-challenged lungs in vivo.

OVA challenge of mice with disrupted NF-κB function such as conditional knockout of IKKB or transgenic IκBα mutant expression selectively in airway epithelium recovered significantly less IL-4, IL-5, and IL-13 in the BAL fluid (7, 28). Th2 cytokines, including IL-4, IL-5, IL-13, and IL-33, play an essential role in the pathogenesis of asthma (29, 30). We observed a major drop in BAL fluid levels of IL-4, IL-5, IL-13, and IL-33 in Rip2 siRNA-treated OVA-challenged mice. There is increasing evidence supporting the role of IL-1β, expressed in a NF-κB–dependent manner and activated by Nod-like receptor protein-3 inflammasome in allergic airways, in inducing IL-5, IL-13, IL-17, IL-33, and TSLP production in asthma (30, 31). Rip2 gene silencing strongly halted the rise of IL-1β BAL fluid level as well as IL-17, IL-33, and TSLP expression in lung tissues. IL-33 is capable of enhancing the IL-5 and IL-13 production through NF-κB activation by Th2 cells (32). IL-17 plays a critical role in neutrophil and eosinophil recruitment to the lungs in severe asthma (30). Taken together, Rip2 gene silencing can suppress a wide spectrum of proinflammatory cytokines in experimental mouse asthma and is likely via disruption of NF-κB pathway. Furthermore, our present data show that the anti-inflammatory effect of S2 is at least in part mediated through a suppressive action on T lymphocytes, as OVA-specific IL-4, IL-5, and IL-13 productions were reduced in thoracic lymph node cultures from Rip2 siRNA-treated mice. The reduction in Th2 cytokine production in these cultures was accompanied by an increase in the production of IFN-γ.

Our findings show that Rip2 gene silencing prevented inflammatory cell infiltration into the airways, as evidenced by a significant drop in total and eosinophil counts in BAL fluid, and in tissue eosinophilia in lung sections. Eosinophil transmigration into the airways is orchestrated by Th2 cytokines IL-5 and IL-13 as well as TNF-α, and coordinated by specific chemokines eotaxin and RANTES in combination with adhesion molecules such as ICAM-1, VCAM-1, and E-selectin (34). IL-13 and IL-17 have been shown to induce eotaxin production from airway epithelial and smooth muscle cells, respectively (35, 36). TNF-α can upregulate the epithelial expression of ICAM-1 and VCAM-1 (37). Besides, combination of IL-17 and TNF-α synergistically induced RANTES,
E-selectin, and ICAM-1 expressions in human endothelial cells (38). IL-33 has been shown to enhance the differentiation and survival of eosinophils (39). Intratracheal Rip2 siRNA strongly suppressed eotaxin and RANTES, TNF-α, ICAM-1, VCAM-1, and E-selectin in OVA-challenged lungs. These results are most likely due to interruption of NF-κB transcriptional activity by Rip2 knockdown, as the genes for eotaxin, TNF-α, RANTES, VCAM-1, and E-selectin contain the κB site for NF-κB within their promoters (40).

There is concrete evidence that goblet cell hyperplasia and Muc5ac production require IL-4, IL-5, and IL-13 (41). Muc5ac gene expression is dependent on the transcriptional activity of NF-κB (40, 41). Selective ablation of NF-κB function in airway epithelial cells (41-43). As such, the marked decrease in VCAM-1, and E-selectin contain the κB (7). Recent studies revealed the important role of IL-1 likely due to interruption of NF-κB transcription factor (44). Our results show that Rip2 gene silencing markedly reduced NF-κB pathway in airway epithelium.

Elevated serum IgE levels are a hallmark of the Th2 immune response. NF-κB plays a crucial role in B cell proliferation and development (4). In addition, IL-4 and IL-13 are important in directing B cell growth, differentiation, and secretion of IgE (4, 29). The biological activities of IgE are mediated through its interaction with the FcεRI on mast cells and basophils. Cross-linking of FcεRI initiates multiple signaling cascades leading to NF-κB activation and production of lipid mediators, cytokines, and chemokines (3, 4). Our data showed that Rip2 siRNA (6 doses) given at the later stage of OVA sensitization and during OVA challenge substantially reduced serum levels of total IgE and OVA-specific IgE. The observed reduction may be contributed by its disruptive action on NF-κB activation in B cells, and on IL-4- and IL-13-mediated class switching to IgE.

Increased exhaled NO is associated with increased iNOS expression in the lung epithelium of asthma patients (44). IL-13, IL-1β, and TNF-α have been shown to induce iNOS expression in human bronchial epithelial cells, leading to elevated NO production (45, 46). In addition, iNOS gene expression is regulated by the NF-κB pathway (40). Our results show that Rip2 gene silencing markedly suppressed the OVA-induced iNOS expression in the lungs, which may be due to the direct interruption of NF-κB signaling and the reduced level of IL-13, IL-1β, and TNF-α in the allergic airways.

IL-4, IL-5, IL-13, TNF-α, IL-17, and IL-33 have been implicated to play a role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory mediators such as major basic protein and cytoelastic leukotrienes, which are closely associated with AHR development (34, 37, 47–50). Inhibition of NF-κB has also been shown to attenuate AHR in experimental asthma (19, 51). Rip2 gene silencing strongly suppressed methacholine-induced AHR, which may be resultant from the major drop in proinflammatory cytokines and tissue eosinophilia by Rip2 siRNA treatment.

Although we have demonstrated the protective effects of Rip2 gene silencing on allergic airway inflammation, the upstream molecule(s) responsible for the activation of Rip2 remains to be determined. OVA-induced mouse asthma is a complex model of Th2 immune response involving a wide panel of proinflammatory mediators. These mediators can activate proteins upstream of Rip2 upon OVA immunization and challenge. In addition to Nod1/2 proteins being critical for Rip2 activation (26, 27), IL-1R (10), IL-18R (10), TCR (11), TLR-3 (52), and Nod-like receptor protein-10 (53, 54) can also be activated in OVA-induced airway inflammation, leading to Rip2 stimulation and the downstream NF-κB activation. Therefore, it is likely that OVA acts through multiple upstream pathways to stimulate Rip2 activity.

To our knowledge, we report in this study for the first time that lung Rip2 level was substantially elevated in experimental asthma, and Rip2 gene silencing markedly reduced lung Rip2 level and attenuated OVA-induced airway inflammation and AHR, probably via disruption of the NF-κB signaling pathway. Our findings strongly implicate Rip2 as a novel therapeutic target for the treatment of asthma.

Disclosures
The authors have no financial conflicts of interest.

References


