

A20 Attenuates Allergic Airway Inflammation in Mice¹

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TNF receptor 1 can activate signaling pathways leading to the activation of NF- κ B. A20, an NF- κ B-inducible protein, negatively regulates these signaling pathways and acts as an anti-inflammatory mediator. Therefore, A20 is viewed as a potential therapeutic target for inflammatory disease. In this study, we examined the effect of A20 on an OVA-induced allergic airway inflammation model in mice. We used an adenovirus containing A20 cDNA (Ad-A20) that was delivered intratracheally before OVA challenge. Single administration of Ad-A20 reduced airway inflammatory cell recruitment and peribronchiolar inflammation and suppressed the production of various cytokines in bronchoalveolar fluid. In addition, Ad-A20 suppressed mucus production and prevented the development of airway hyperresponsiveness. The protective effect of Ad-A20 was mediated by the inhibition of the NF- κ B signaling pathway. Taken together, our results suggest that the development of an immunoregulatory strategy based on A20 may have therapeutic potential for the treatment of allergic asthma. *The Journal of Immunology*, 2009, 183: 0000–0000.

Bronchial asthma is a common disease of the respiratory system with a rapidly rising prevalence in industrialized countries. It is defined as a chronic inflammatory disease characterized by reversible airway obstruction in response to allergens, chronic eosinophilic airway inflammation, and nonspecific airway hyperresponsiveness (AHR)⁴ (1). Various regimens including bronchodilators and anti-inflammatory drugs are prescribed in combination for the treatment of bronchial asthma. Among them, therapy using inhaled corticosteroids is recognized as one of the most important strategies because it is highly effective in controlling asthma symptoms and preventing acute exacerbations (2). Nevertheless, there is still a subgroup of patients who have severe asthma that is resistant to corticosteroid therapy; this subtype of bronchial asthma is termed difficult-to-control or refractory asthma (3, 4). These corticosteroid-resistant asthma patients are characterized by elevated levels of TNF- α (5, 6) and neutrophils (6, 7), which can be treatment targets.

TNF- α is emerging as a potent target for the treatment of asthma irrespective of severity. Before the introduction of anti-TNF- α therapy, there were many attempts to develop strategies to complement or replace the preexisting regimens. Based on the observation that type 2 helper T cell (Th2) cytokines (IL-4, 5) and IgE

contribute to the induction of allergic inflammation in asthma, compounds targeting IL-4, 5, or IgE have been extensively investigated. However, results from serial studies using anti-IL-4 or anti-IL-5 Abs were disappointing (8). Furthermore, although blockade of IgE using a mAb (omalizumab) is efficacious, its use is limited by the level of total serum IgE and the body weight of the patient (9, 10). Therefore, to develop new asthma therapies, blockade of nonspecific proinflammatory cytokines including TNF- α (8) has been investigated. In chronic inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, and sarcoidosis in which type 1 helper T cell cytokines such as TNF- α and IFN- γ dominate, blockade of TNF- α using a specific mAb (infliximab) or soluble TNF- α receptor fusion protein (etanercept) results in disease remission (11). Infliximab (12, 13) and etanercept (14, 15) have been shown to be effective in a murine model of asthma and human asthma patients. However, the clinical application of infliximab is limited because of the occurrence of neutralizing Abs (16, 17). In addition, development of tuberculous uveitis (18), sarcoidosis (19), and granulomatous thyroiditis (20) have also reported during etanercept therapy, though there is no production of neutralizing Ab. Thus, anti-TNF- α therapy still requires improvement.

The binding of TNF- α and its related cytokines to their corresponding receptors induces NF- κ B activation (21). TNF- α binds to two distinct receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2, with most of its effects mediated by TNF-R1. Upon binding of TNF- α to TNF-R1, the receptor is able to recruit the cytoplasmic death domain-containing signaling molecule known as TNF receptor-associated death domain (22, 23). TNF receptor-associated death domain in turn recruits the death domain-containing adapter proteins, FAS-associated death domain, receptor interacting protein (RIP), and TNF-associated factor 2 (TRAF2) into the receptor complex. Recruitment of FAS-associated death domain to the receptor complex leads to cell death, whereas recruitment of RIP and TRAF2 contributes to NF- κ B activation via recruitment and phosphorylation of I κ B kinase (22). TNF-R1-mediated NF- κ B activation is controlled by both positive and negative regulatory loops. The zinc protein A20 is encoded by an immediate early response gene and acts as a potent inhibitor of NF- κ B signaling pathway (24). Interestingly, the expression of A20 is itself under the control of NF- κ B, suggesting that A20 is involved in the negative feedback

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⁴ Abbreviations used in this paper: AHR, airway hyper-responsiveness; TNF-R1, TNF receptor 1; RIP, receptor interacting protein; TRAF2, TNF-associated factor 2; i.t., intratracheal; BAL, bronchoalveolar lavage; BALF, BAL fluid; Ad-A20, adenovirus expressing A20; Ad-GFP, adenovirus expressing GFP.

regulation of NF- κ B activation (24). Prevention of NF- κ B activation by overexpression of A20 has been reported in various systems including pancreatic β -cells (25) and myocardial cells (26). However, the effect of A20 in asthma has not yet been reported. Therefore, we performed this study to examine the effectiveness of A20 for reducing airway inflammatory reactions and improving asthma symptoms in an OVA-induced airway inflammation model.

Materials and Methods

Animals

Pathogen-free female BALB/c mice were obtained from Samtaco, housed in a laminar flow cabinet, and maintained on standard laboratory chow ad libitum. Mice were 7–8 wk old at the start of each experiment. All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee at Chonbuk National University.

Preparation of adenovirus

Preparation and amplification of adenovirus was performed as described previously (27). Adenoviruses encoding A20 were created using the ViraPower adenovirus expression system according to the manufacturer's instruction (Invitrogen). Site specific recombination between entry vectors (A20-pENTR) and the adenoviral destination vector (pAd/PL-DEST) were established with LR clonase II (Invitrogen). Viruses from the culture supernatants of 293 cells that showed cytopathogenic effects were purified by cesium chloride banding. Virus titers were determined by a plaque assay using serial dilution.

Immunization, challenge, and adenoviral delivery in lung tissue

Mice were immunized i.p. with 20 μ g of OVA (grade V; Sigma-Aldrich) plus 2.25 mg aluminum hydroxide adjuvant on day 0 and OVA alone without alum on day 14. The immunized mice were exposed to aerosolized OVA on days 28, 29, and 30. Aerosolization of OVA was performed using a chamber that was adapted for mice. Animals were exposed to OVA (4%) using an ultrasonic nebulizer (NE-U12, Omron; output 0.8 ml/min) for 20 min in a Plexiglas exposure chamber (24.5 $\times$ 40.5 $\times$ 15.0 cm). Control animals received the same immunization, but were exposed to aerosolized saline instead of OVA during airway challenge. To examine the therapeutic effects of A20, the mice received a single intratracheal (i.t.) delivery of 30 μ l of adenovirus (2×10^9 pfu) 3 days before the first OVA challenge.

Bronchoalveolar lavage (BAL)

BAL was performed at the time indicated after the last airway challenge. Mice were anesthetized and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged with 0.7 ml of PBS. The BAL fluid (BALF) samples were collected and the number of cells in a 100 μ l aliquot was determined using a hemocytometer. The remaining sample was centrifuged, and the supernatant was stored at -70°C until cytokine assays were performed. The pellet was resuspended in PBS and a cytospin preparation of BAL cells was stained with Diff-Quik (International Reagents). The different cell types were enumerated based on their morphology and staining profile.

Determination of AHR

AHR was assessed as a change in airway function after challenge with aerosolized methacholine via the airway, as described previously (28). Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected i.p. The trachea was then exposed through midcervical incision and tracheostomized, and an 18-gauge metal needle was then inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ), and each mouse was quasi-sinusoidally ventilated with a nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume similar to that occurring during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to a water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with increasing concentrations of methacholine (2.5–50 mg/ml in saline) in an aerosol form. The data needed to calculate R_L was collected continuously following each methacholine challenge. Maximum R_L values were selected to express changes in airway function, which was represented as the percent change from baseline after saline aerosol treatment.

Cytokine assays

TNF- α , IL-5, IL-13, eotaxin, ICAM-1, and VCAM-1 levels in BAL were determined by ELISA (R&D Systems). The lower limits of detection for the cytokines were as follows: TNF- α (>5.1 pg/ml), IL-5 (>5 pg/ml), IL-13 (>1.5 pg/ml), eotaxin (>3 pg/ml), ICAM-1 (>0.017 ng/ml), and VCAM-1 (>20 pg/ml).

EMSA

Nuclear extracts were prepared from the lung tissues as described previously (29). To inhibit endogenous protease activity, 1 mM PMSF was added. An oligonucleotide containing the κ -chain binding site (κ B, 5'-CCGGTTAACAGAGGGGGCTTCCGAG-3') was synthesized and used as a probe for the gel retardation assay. The two complementary strands were then annealed and labeled with [α - ^{32} P]2'-deoxycytidine 5'-triphosphate. Labeled oligonucleotides (10,000 cpm), 10 μ g of nuclear extracts, and binding buffer (10 mM Tris-HCl (pH 7.6), 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI $\cdot$ dC), 1 mM DTT) were then incubated for 30 min at room temperature in a final volume of 20 μ l. Next, the reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in a 0.5 $\times$ Tris-borate buffer, and the gels were then dried and examined by autoradiography.

Western blot analysis

Lung tissues were homogenized with protease and phosphatase inhibitors and prepared in protein extraction solution (PRO-PREP, iNtRON). The homogenates, which contained 30 μ g of protein, were separated by 10% SDS-PAGE, and transferred to nitrocellulose sheets. The blot was probed with 1 μ g/ml primary Abs for RIP (Cell Signaling Technology), p50, p65, I κ B α , proliferating cell nuclear Ag, β -actin (Santa Cruz Biotechnology), or A20 (Calbiochem). Alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as a secondary Ab.

Histological studies and mucin analysis

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at 4 μ m, placed on glass slides, and deparaffinized. Sections were stained with H&E for light microscopic examinations. For immunohistochemistry, lungs were removed 3 days after adenovirus administration, and sections were subjected to immunostaining using the anti-A20 Ab. For the detection of mucin, tissues were stained with Alcian blue/periodic acid-Schiff and counterstained with H&E. For quantification of mucin levels, BALF was collected, and cells were removed by centrifugation. Lung mucin levels were measured using the mucin-binding lectin, jacalin (Calbiochem). Wells of microtiter plates (Immulon IV; Fisher Scientific) were precoated with serial 2-fold dilutions of mucin standard (Sigma-Aldrich) and samples were diluted in PBS for 2 h at 37°C and then blocked with 0.2% I-block (Applied Biosystems) in PBS containing 0.05% Tween 20. Plates were washed with PBS containing 0.05% Tween 20 and then incubated with 5 μ g/ml biotinylated jacalin (Vector Laboratories) diluted in PBS for 1 h at 37°C . Plates were washed and incubated with a 1/500 dilution of streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. After a final wash, plates were developed with *p*-nitrophenyl phosphate diluted in alkaline buffer solution, and absorbance was measured at 405 nm.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Fisher test. The significance of differences between groups was determined using Student's unpaired *t* test. A value of $p < 0.05$ was accepted as an indication of statistical significance.

Results

Adenoviral-mediated expression of A20 in the mouse lung suppresses OVA-induced chemotaxis and inflammatory reactions

To determine the therapeutic effect of A20 on airway inflammatory reactions and asthma symptoms in an OVA-induced airway inflammation model, an adenovirus expressing A20 under the control of a cytomegalovirus promoter (Ad-A20) was used. Airway inflammation was induced in BALB/c mice by i.p. administration of OVA followed by challenges with aerosolized OVA on days 28, 29, and 30 (Fig. 1). As a negative control, mice were treated with saline. To express A20 in airway, mice were injected with 2×10^9

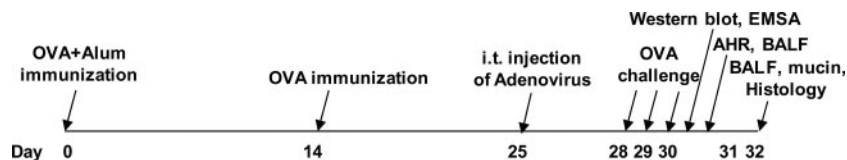


FIGURE 1. Schematic diagram of the experimental protocol. Mice were sensitized and challenged with OVA or saline on day 0, 14, 28, 29, and 30. Ad-GFP or Ad-A20 was injected i.t. 3 days before the first OVA challenge. Western blotting and EMSA analyses for NF- κ B activation (1 h), AHR (10 h), cytokines assay in BALF (2 and 24 h), differential cell counts in BALF (48 h), and mucin analysis and end-point histology (48 h) were performed after the last OVA challenge.

pfu of either Ad-A20 or control (adenovirus expressing GFP, Ad-GFP) virus i.t. 3 days before the first OVA challenge. We collected lung tissues at several time points after adenoviral injection and analyzed the A20 expression in lung homogenates. Western blot analysis confirmed the highest expression of A20 at day 1 in lung tissue of Ad-A20-injected mice, but not in Ad-GFP-injected mice and then slowly declined over the next days (Fig. 2A). To determine the cellular localization of A20 gene expression in the lungs, we performed separate experiments in which mice were injected with either Ad-A20 or Ad-GFP virus. Three days after virus administration, A20 expression was analyzed by immunohistochemistry. Mice injected with Ad-A20 showed immunoreactivity for A20 in bronchiolar and alveolar epithelial cells (Fig. 2B). However, A20 expression was not detectable in lung sections from saline-treated control and Ad-GFP-injected mice.

To examine the effect of A20 on chemotaxis, that is, recruitment of inflammatory cells into airway, inflammatory cells were counted in BALF. We analyzed the cellular composition of the BALF of mice 48 h after the last OVA challenge. In the saline-treated mice, OVA challenge resulted in a marked increase of eosinophils and slight increases of macrophages and lymphocytes when compared

with control mice (Fig. 2C). However, prior injection with A20 significantly attenuated OVA challenge-induced recruitment of eosinophils ($p < 0.01$). The observed reduction in chemotaxis into the airway correlated with the histological changes of lung parenchyma. Lungs from OVA-challenged mice treated with saline showed widespread perivascular and peribronchiolar inflammatory cell infiltrates (Fig. 2D). However, prior injection with Ad-A20 resulted in a significant reduction of inflammatory cell infiltration, whereas administration of Ad-GFP had no detectable effect. These results indicate that treatment with A20 efficiently inhibits the infiltration of inflammatory cells and attenuates allergic airway inflammation.

A20 decreases NF- κ B activation through down-regulation of RIP

Based on the knowledge that NF- κ B plays a key role in allergic inflammation of lung by inducing the transcription of various proinflammatory mediators (30) and that A20 is involved in the negative feedback regulation of NF- κ B (24), we hypothesized that the adenovirus-mediated expression of A20 would attenuate airway inflammatory reactions by suppressing NF- κ B activation. To

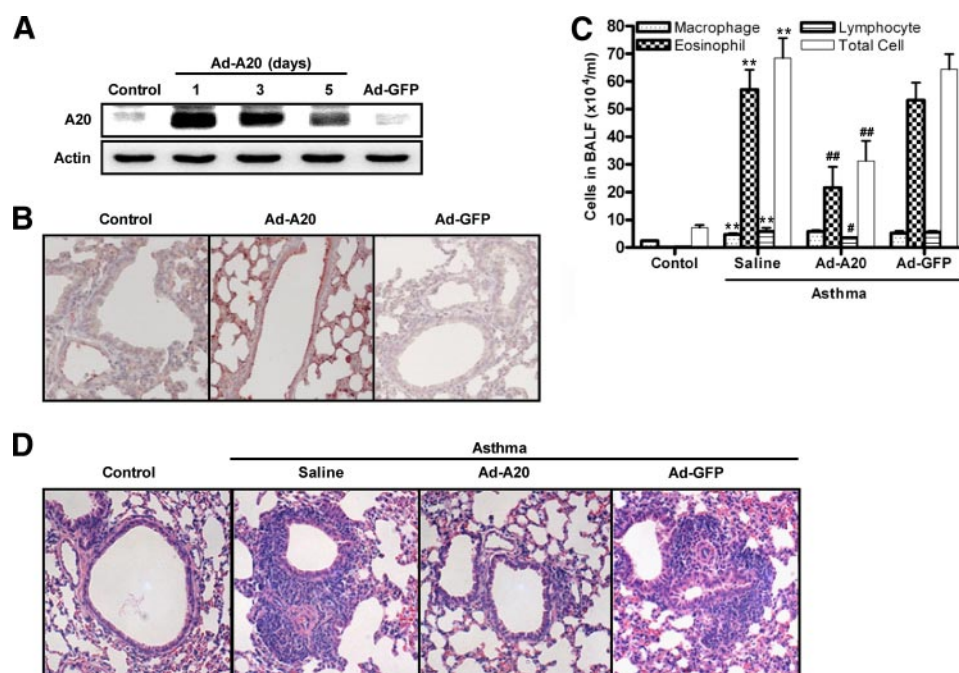


FIGURE 2. A20 expression, differential cell counts in BALF and histological evaluation of lung inflammation following OVA sensitization and treatment with Ad-A20. *A*, Mice were injected with 2×10^9 pfu of either Ad-A20 or Ad-GFP virus i.t. Lung homogenates prepared at indicated time points after viral injection were subjected to Western blot analysis with anti-A20 Ab. *B*, Three days after i.t. virus injection, lungs were removed and fixed in 10% formalin. Paraffin-embedded sections were immunostained with anti-A20 Ab. Brown stainings in bronchiolar and alveolar epithelial cells reveal the presence of A20. *C*, Mice were injected with Ad-A20 or Ad-GFP 3 days before the first OVA challenge. The effect of A20 on the OVA challenge-induced differential cell counts in BALF was analyzed at 48 h after the last OVA challenge. Results from five independent experiments with three mice/group are given as mean $\pm$ SEM. **, $p < 0.01$ vs untreated control; #, $p < 0.05$; ##, $p < 0.01$ vs saline-treated group. *D*, Paraffin-embedded lung sections were prepared 48 h after the last OVA challenge and stained with H&E. Data are representative of three independent experiments.

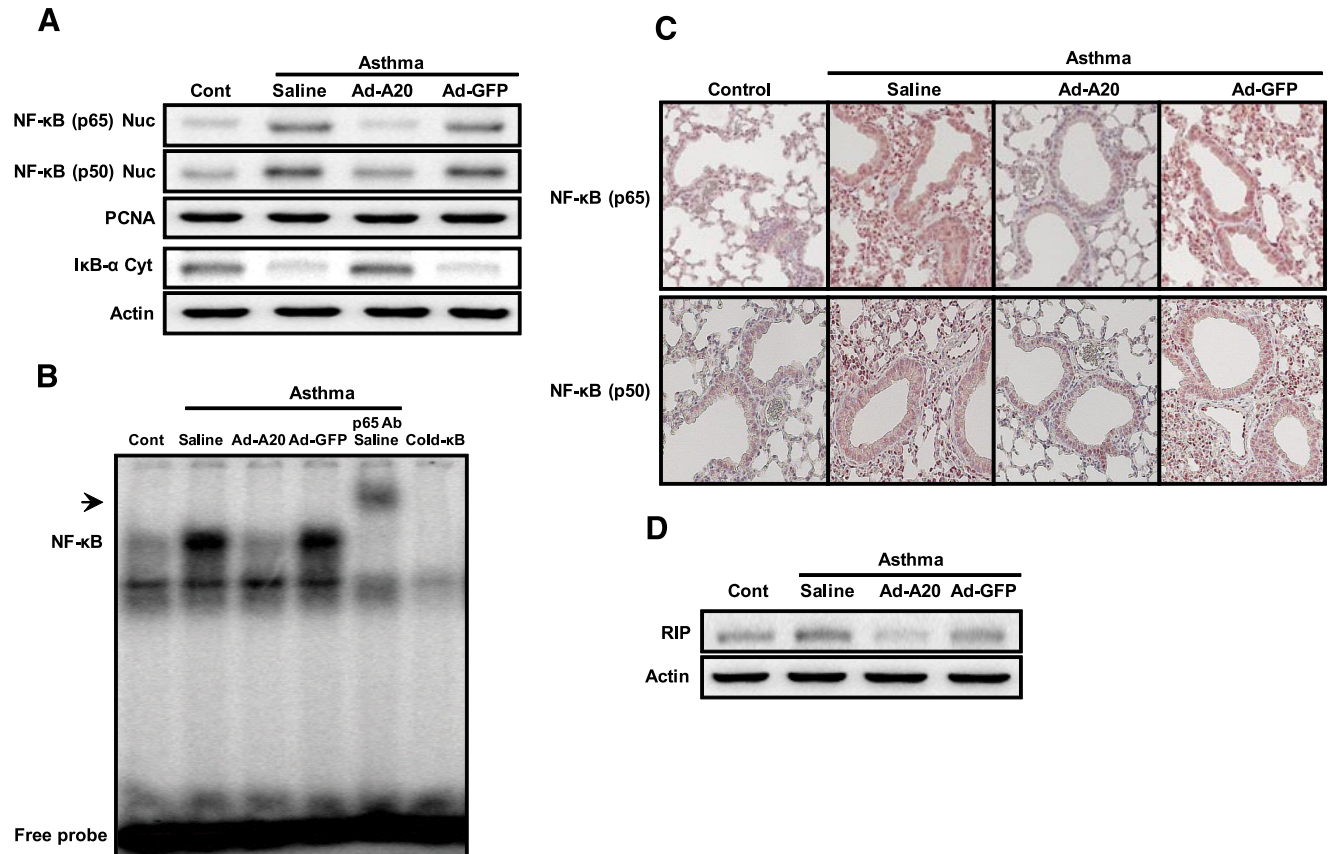


FIGURE 3. Effect of A20 on OVA challenge-induced NF-κB activation and protein level of RIP. Mice were injected with Ad-A20 or Ad-GFP 3 days before the first OVA challenge. Lung homogenates were prepared 1 h after the last OVA challenge and the translocation of p65 and p50 to the nucleus and IκBα degradation in cytoplasm (A) and NF-κB DNA binding activity (B) were assessed by Western blotting and EMSA, respectively. Actin and proliferating cell nuclear Ag were used as loading controls for cytosolic and nuclear proteins, respectively. Small arrow indicates supershifted complexes. C, Nuclear translocation of the p65 and p50 subunits of NF-κB was examined by immunohistochemistry staining. Representative photomicrographs are shown. Magnification, $\times 200$. D, The change of RIP after OVA challenge was evaluated by Western blotting. Similar results were obtained from three independent experiments.

address this issue, we first studied the nuclear translocation and DNA binding activity of NF-κB in lung tissues after OVA challenge. There was an increase in the level of the p50 and p65 subunits of NF-κB in the nuclei from lung tissues of OVA-challenged mice treated with saline (Fig. 3A), as well as an increase in the binding activity of lung nuclear extracts to a NF-κB consensus sequence (Fig. 3B) compared with control mice. DNA binding of NF-κB was resolved by supershift analysis to comprise the p65 subunit (Fig. 3B, lane 5). Specificity of the DNA-protein interactions for NF-κB was demonstrated by competition assays using a 50-fold excess of unlabeled oligonucleotide (Fig. 3B, lane 6). Immunostaining of p50 and p65 subunits in lung tissues also confirmed the nuclear translocation of p50 and p65 subunits (Fig. 3C). However, nuclear extracts prepared from Ad-A20-injected mice showed suppressed nuclear translocation and NF-κB DNA binding. We also examined the alteration of IκBα levels in the cytosol fraction. Lung tissues from OVA-challenged mice treated with saline showed a decreased level of IκBα protein in the cytosol when compared with a similar fraction of control lung, but the increased IκBα degradation as a result of the OVA challenge was markedly suppressed by Ad-A20 injection (Fig. 3A).

We next determined the protein level of RIP because A20 is known to regulate NF-κB via RIP (22, 24). To test the hypothesis that A20 inhibits NF-κB activation by suppressing RIP, total lysates prepared 24 h after the last OVA challenge were analyzed by Western blotting (Fig. 3D). In saline-treated mice, OVA challenge

increased the protein level of RIP, whereas prior injection with Ad-A20 markedly decreased the protein level of RIP. These results suggest that the probable mechanism by which A20 inhibits OVA challenge-induced NF-κB activation is by down-regulating the protein level of RIP.

A20 reduces the levels of cytokines involved in the pathophysiology of asthma in BALF

The experiments presented in Figs. 2 and 3 suggest that signaling via the TNF-α-TNFR-1 axis is important for the inflammatory reaction in the airway. Furthermore, TNF-α is known to mediate the pathophysiology of severe asthma (31, 32). Therefore, the levels of TNF-α in BALF were determined using an ELISA kit. As shown in Fig. 4A, OVA challenge induced a 15.7-fold increase in TNF-α ($p < 0.01$). Prior injection with Ad-A20 significantly diminished OVA challenge-mediated TNF-α production ($p < 0.05$). In contrast, Ad-GFP failed to reduce TNF-α production (Fig. 4A). To test whether T cell responses were affected by A20, the levels of Th2 cytokines were measured. OVA challenge resulted in a 7.2- and 9.7-fold increase in the levels of IL-5 and IL-13 in BALF, respectively. However, A20 significantly diminished the OVA challenge-mediated increase in IL-5 and IL-13 by 59.5% and 50.1%, respectively (Fig. 4B). In addition, chemotactic cytokine for the recruitment of eosinophils (eotaxin) was assayed. Eotaxin level in the BALF of mice preinjected with Ad-A20 was significantly lower than those in the saline or Ad-GFP groups (Fig. 4C). This

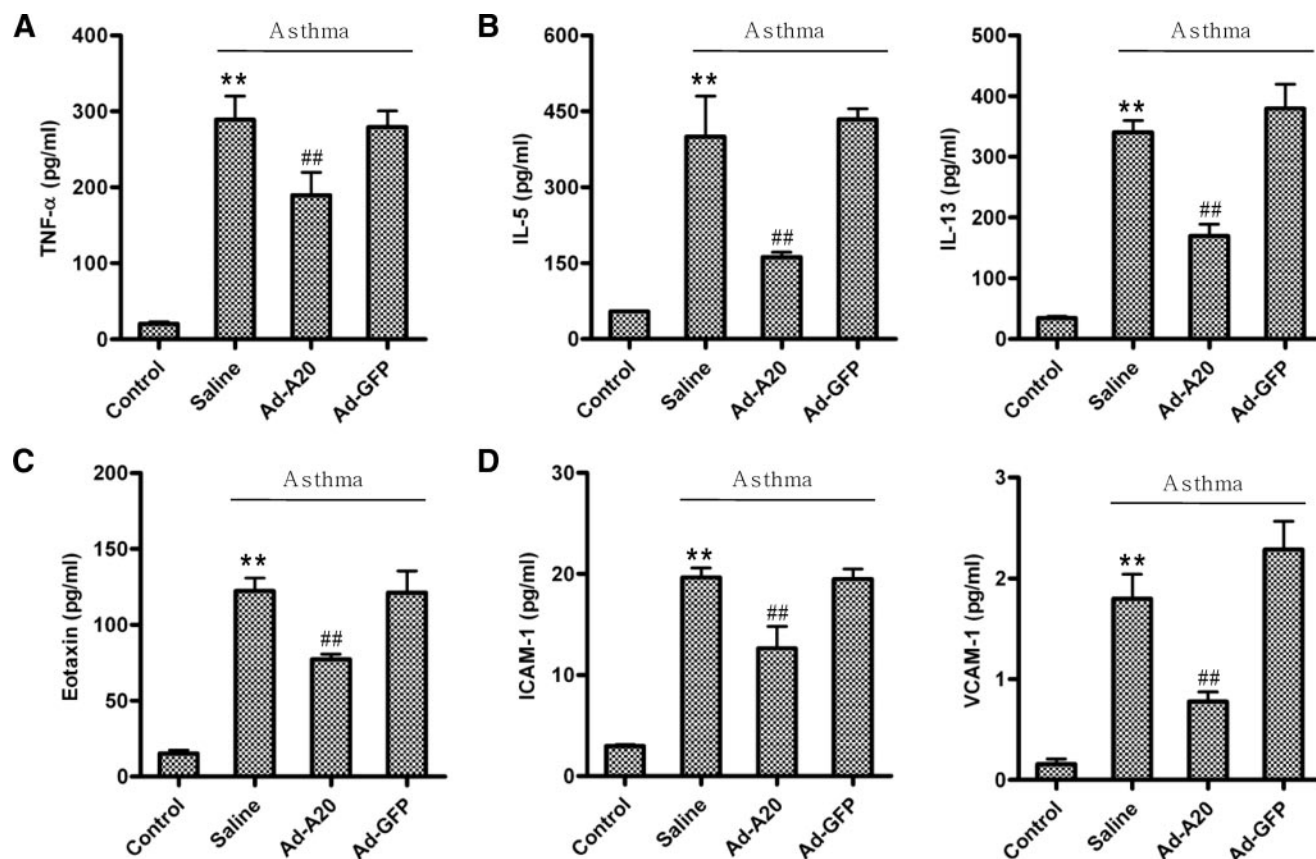


FIGURE 4. Assessment of proinflammatory cytokines in the BALF of OVA-sensitized mice treated with Ad-A20. Various cytokines in BALF were measured by specific ELISAs. The level of TNF- α , was determined 2 h after the last OVA challenge, and the levels of IL-5, IL-13, eotaxin, VCAM-1, and ICAM-1 were determined 24 h after the last OVA challenge. The results of three independent experiments with three mice/group are expressed as mean $\pm$ SEM. **, $p < 0.01$ vs untreated control; ##, $p < 0.01$ vs saline-treated group.

result was consistent with the reduced number of eosinophils in the BALF of Ad-A20 injected mice (Fig. 2). Finally, changes in the levels of NF- κ B-dependent cytokines (ICAM-1 and VCAM-1)

were similar to those seen for the aforementioned cytokines, indicating that the OVA challenge-induced increase in cytokine levels can be reversed by A20 (Fig. 4D).

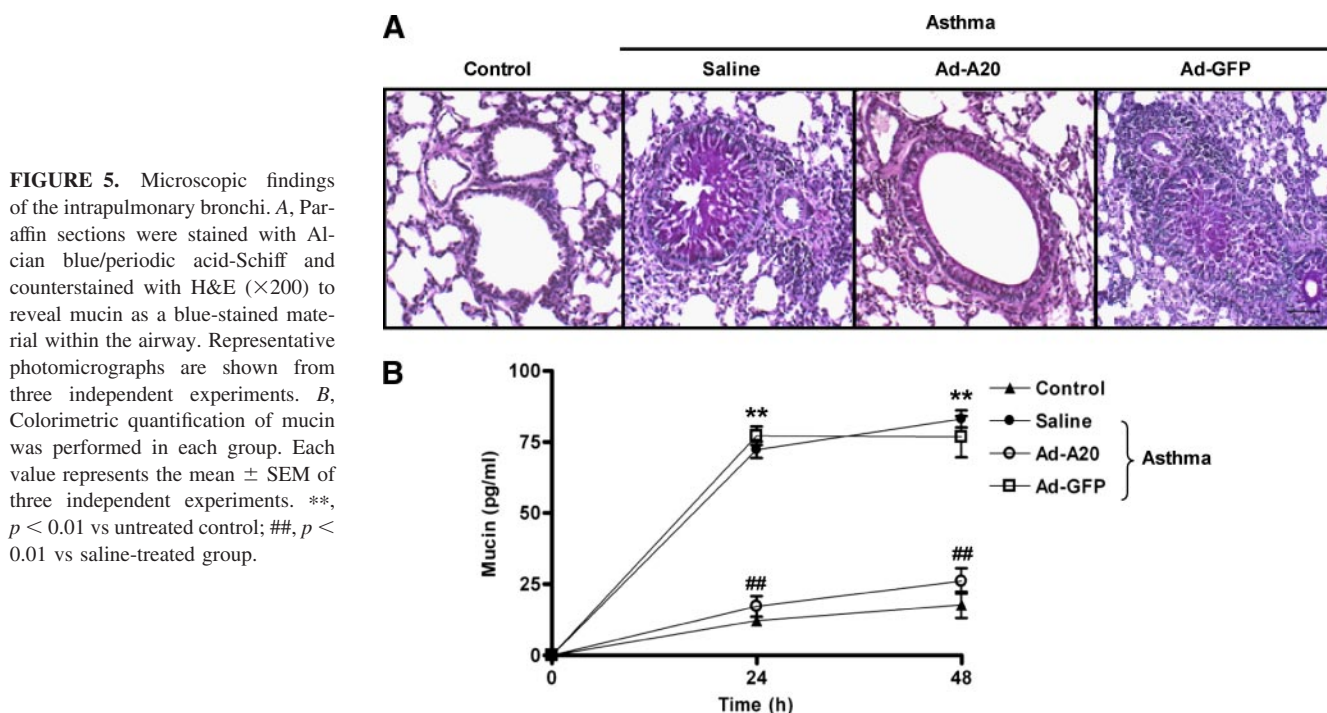


FIGURE 5. Microscopic findings of the intrapulmonary bronchi. A, Paraffin sections were stained with Alcian blue/periodic acid-Schiff and counterstained with H&E ($\times 200$) to reveal mucin as a blue-stained material within the airway. Representative photomicrographs are shown from three independent experiments. B, Colorimetric quantification of mucin was performed in each group. Each value represents the mean $\pm$ SEM of three independent experiments. **, $p < 0.01$ vs untreated control; ##, $p < 0.01$ vs saline-treated group.

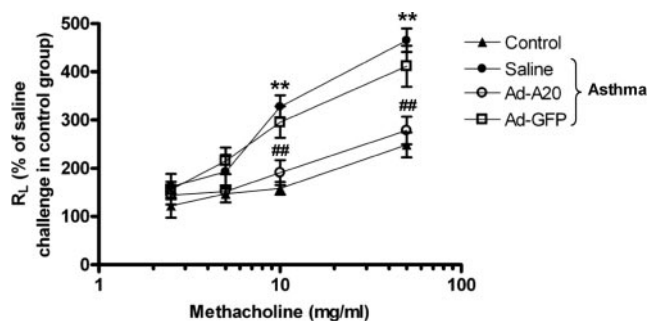


FIGURE 6. AHR responses in OVA-sensitized mice treated with Ad-A20. All animals were sensitized to OVA as described in Fig. 1 and nebulized with various concentrations of methacholine (2.5, 5, 10, 50 mg/ml) as a bronchoconstrictor. Data are shown as airway resistance (cm H₂O per sec per ml) and percent reduction where reducing resistance to that of the vehicle-treated nonasthmatic control group would equal to 100%. Each value represents the mean $\pm$ SEM of three independent experiments. **, $p < 0.01$ vs control; ##, $p < 0.01$ vs saline-treated group.

A20 attenuates excessive production and secretion of mucin in the airway

Bronchiolar mucin accumulation, an additional hallmark most commonly associated with the chronic asthmatic response, can contribute to airway obstruction, dyspnea, and cough in asthma patients (33). We therefore evaluated whether A20 affects mucin production from bronchial goblet cells. Minimal intrabronchial mucin was observed in control mice, whereas in OVA-challenged mice treated with saline or Ad-GFP, there was excessive mucin content in airway that caused partial or complete obstruction of airway. In contrast, mucin production was effectively attenuated to near normal levels by adenoviral expression of A20 (Fig. 5A). The measured mucin content correlated well with the histology data (Fig. 5B).

A20 prevents the development of AHR

One functional consequence of the inflammatory process that underlies asthma is a hyperresponsiveness to methacholine, a bronchoconstrictor. Furthermore, recent studies have shown that AHR in asthma is caused by TNF- α -induced smooth muscle contraction (28, 34). We therefore examined whether A20 also influenced this endpoint. As shown in Fig. 6, methacholine increased AHR in a dose-dependent manner. However, A20 reduced AHR in asthmatic mice by $\sim 40\%$ ($p < 0.01$) and restored AHR to levels similar to that of the control.

Discussion

The present study was designed to investigate whether negative regulation of NF- κ B by A20 would affect OVA challenge-induced inflammatory responses in the airways. To this end, A20 was delivered as cDNA in an adenoviral vector before sensitization with OVA. The results show that i.t. injection of Ad-A20 inhibits OVA challenge-induced allergic airway inflammation. To the best of our knowledge, the results presented in this study provide the first experimental evidence that A20 inhibits the development of Th2-driven airway inflammation.

We performed initial experiments with adenoviral vector expressing A20 to determine the timing and localization of transgene expression. Using direct intratracheal delivery of Ad-A20, we demonstrated expression of A20 in lung epithelium. A20 expression was detected as early as 24 h after injection and this expression persisted through 5 days. It is interesting that unlike other adenovirus-mediated gene delivery, Ad-A20 maintained its ex-

pression level for 5 days. Previous reports suggest that clearance of adenovirus from the lung is affected by TNF- α (35). Expression of adenovirus-delivered gene is enhanced (36) and in some cases duration of expression is prolonged in mice lacking functional TNF- α or TNF receptors (37). Because A20 is known as a negative regulator of TNF signaling system, A20 expression might be prolonged. In our immunization protocol, Ad-A20 was delivered 3 days before the first OVA challenge and A20 expression was maintained enough levels during the OVA challenge phase.

TNF- α is a critical contributor to allergic asthma, and a number of its properties are related to characteristic features of the disease. These include recruitment of eosinophils, up-regulation of adhesion molecules such as ICAM-1 and VCAM-1, increase of AHR, increase of cytokine release, and induction of corticosteroid resistance (31). Moreover, the concentration of TNF- α in BALF is increased in patients with refractory or severe asthma compared with patients with mild asthma and healthy subjects (38), suggesting that up-regulation of the TNF- α signaling pathway provides the link between the pathological airway response and the development of severe asthma. In addition, the administration of recombinant TNF- α to normal subjects leads to the development of AHR (39). Classically, TNF-R1 activation leads to the recruitment of the adapter molecule, RIP, to the membrane. Therefore, we investigated whether this signaling event was affected by A20. A20 is known to bind to and target this adapter molecule for proteosomal degradation, thereby preventing TNF- α -induced NF- κ B activation (24, 40). Our results clearly show that A20 down-regulates the protein level of RIP, providing a possible mechanistic explanation for the observed effects of A20 on the TNF-R1-mediated inflammatory reactions in the airway. Similarly, A20 has been shown previously to interact with the TRAF2 (41), TRAF6 (42), and NEMO (NF- κ B essential modifier) (43), suggesting that these interactions might also be involved in the A20-mediated inhibition of NF- κ B activation induced by OVA challenge. A20 has both ubiquitinating and deubiquitinating enzymatic activity in the TNF-R1 signaling pathway. A20 contains an N-terminal domain that belongs to the ovarian-tumor superfamily of deubiquitinating cysteine proteases and deubiquitinates K63-linked polyubiquitinated RIP and TRAF2/6, thereby blocking TNF- α -induced NF- κ B signaling. A20 also functions as a ubiquitination enzyme for K48-linked polyubiquitination for proteosomal degradation (22, 40). Although the biological effects of repressed protein levels of RIP by A20 is functionally analogous to a deficiency in recruitment of these signaling molecules to TNF-R1, further studies are required to determine whether A20 directly regulates the aforementioned regulators of NF- κ B signaling pathways.

The transcription factor NF- κ B regulates multiple proinflammatory genes that can contribute to allergic asthma. In an inflammatory setting, cells are required to express a new or inducible set of anti-inflammatory genes as a mechanism to counteract the physiological stresses that otherwise lead to cellular damage. A20, a TNF- α -inducible early response gene product with a potent anti-inflammatory activity, is a typical example of this self-protecting feedback loop (24). In this study, we validated this paradigm in an Ad-A20-injected and OVA-sensitized allergic airway inflammation model. Overexpression of A20 in airways is sufficient to alleviate asthma symptoms and airway inflammatory responses. As expected, A20 significantly inhibited OVA challenge-induced NF- κ B nuclear translocation. This event led to further reduction of the pathological inflammatory response in the airway tissues. The favorable effects of A20 against inflammatory responses have been studied in various other systems. Mutation of a single amino acid of A20 (E627A) has been shown to correlate with increased sensitivity to atherosclerosis in mice (44), whereas overexpression of

A20 is protective against atherosclerosis in mice (45). A20 also protects pancreatic islets from cytokine toxicity (25), and the heart from myocardial infarction (26). These studies underscore the importance of A20 in controlling inflammatory responses.

The overall effect of A20 on inflammation is mediated primarily by down-regulating the synthesis of a number of cytokines. A20 effectively down-regulates eosinophil recruitment as evidenced by decreased levels of eotaxin, eosinophil chemoattractant, and reduces eosinophils in BALF at later stages after OVA challenge. In addition, histologic evaluation reveals that A20 is also able to reduce OVA challenge-induced peribronchiolar inflammation. Given that eosinophilic infiltration of the airway is a characteristic feature of allergic asthma, we speculate that A20 alleviates airway inflammation by decreasing the severity of eosinophilia. IL-5, a product of Th2 cells, has been shown to provide an essential signal for the expansion and mobilization of eosinophils from the bone marrow into the lung after allergen exposure (46). IL-13, another cytokine produced by Th2 cells, is known to induce expression of eotaxin in airway epithelial cells (47). In our experiments, IL-5 and IL-13 levels in the BALF were lower in mice receiving A20 than in saline- or control virus-treated mice. The recruitment of eosinophils into the airways is also regulated by the expression of adhesion molecules such as VCAM-1 and ICAM-1 by endothelial cells. Our results clearly show that OVA challenge-induced VCAM-1 and ICAM-1 expression is reduced by A20. Therefore, it is possible that A20 interferes with the recruitment of inflammatory cells by reducing chemokine levels and suppressing the expression of adhesion molecules.

We also demonstrated that A20 effectively inhibited OVA challenge-induced AHR to methacholine and abrogated the increased mucus accumulation. A possible explanation for this finding may be that A20 reduces the concentration of TNF- α . AHR could be caused by a direct effect of TNF- α on airway smooth muscle. Studies have demonstrated that incubation of tracheal tissues with TNF- α increases the contractile responses to methacholine or other bronchoconstrictors (48, 49). An alternative mechanism by which A20 could modulate the AHR response is through the down-regulation of Th2 responses. In mice and human, Ag-induced AHR results from Th2-dependent allergic responses, and Th2-associated cytokines such as IL-4, IL-5, and IL-13 are involved in the induction of AHR and mucus secretion (50). Therefore, down-regulation of TNF- α and Th2 cytokines by A20 results in reduced AHR and suppressed mucus accumulation in the airway.

In summary, we have demonstrated that A20 has a potent protective effect in a allergic airway inflammation model. Prior injection of Ad-A20 before OVA challenge reduced the inflammatory cell infiltration, attenuated excess mucin production, and improved the functional airway response to methacholine. Our data suggest that the protective effect of A20 results from the inhibition of the NF- κ B signaling pathway. The results of this study provide novel insight into the disease mechanism underlying asthma and demonstrate that A20 is a promising new candidate for the treatment of allergic asthma.

Disclosures

The authors have no financial conflict of interest.

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