

Adenoviral Gene Transfer of the NF- κ B Inhibitory Protein ABIN-1 Decreases Allergic Airway Inflammation in a Murine Asthma Model*

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Airway inflammation is a characteristic of many lung disorders, including asthma and chronic obstructive pulmonary disease. Using a murine model of allergen-induced asthma, we have demonstrated that adenovirus-mediated delivery of the nuclear factor- κ B (NF- κ B) inhibitory protein ABIN-1 to the lung epithelium results in a considerable reduction of allergen-induced eosinophil infiltration into the lungs. This is associated with an ABIN-1-induced decrease in allergen-specific immunoglobulin E levels in serum, as well as a significant reduction of eotaxin, interleukin-4, and interleukin-1 β in bronchoalveolar lavage fluid. These findings not only prove that NF- κ B plays a critical role in the pathogenesis of allergic inflammation but also illustrate that inhibiting NF- κ B could have therapeutic value in the treatment of asthma and potentially other chronic inflammatory lung diseases.

Bronchial asthma is one of the most common chronic diseases in modern society. It is a general health problem in several industrialized countries and is predicted to remain one for the next decades. With regard to asthma pathogenesis, our understanding has increased tremendously over the last two decades. Therefore, the potential for specifically targeted and constructed therapies has become evident. The onset, development, and clinical manifestations of asthma are driven by a deregulated immune response to an antigen that provokes the activation of CD4⁺ T helper-2 (Th2)¹ lymphocytes and the production of specific cytokines (including interleukin (IL)-1, IL-4, IL-5, and IL-13) (1, 2). Cytokine and chemokine expression results in the recruitment of eosinophils, leading to chronic airway inflammation. Binding and subsequent antigen-

dependent cross-linking of IgE bound to its receptor results in eosinophil and mast cell activation, leading to the local release of mediators, mucus hyperproduction, airflow obstruction, and airway hyperresponsiveness. Moreover, bronchial epithelial cells are able to release various mediators, including chemokines, to initiate inflammatory immune responses (3). Recent studies have demonstrated the expression of the chemokine eotaxin in bronchial epithelium as well as its increase in asthmatic patients (4). This chemokine is a specific chemoattractant for eosinophils and causes selective infiltration of these cells into the lung (5).

The transcription factor nuclear factor- κ B (NF- κ B) plays a pivotal role in immune and inflammatory responses through the regulation of the expression of several proteins, including pro-inflammatory cytokines, chemokines, and adhesion molecules. Uncontrolled activation of the NF- κ B pathway is involved in the pathogenesis of several chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease (6). Several lines of evidence suggest that NF- κ B also plays a central role in the pathogenesis of asthma (7). Activated NF- κ B has been identified in the airways of asthmatic patients, and agents such as allergens, ozone, and viral infections, which are associated with exacerbation of asthma, stimulate activation of NF- κ B. In addition, the major effective treatment for asthma, corticosteroids, is a potent blocker of NF- κ B activation. Finally, mice lacking the NF- κ B p50 or c-Rel subunits develop less airway inflammation on antigen challenge (8, 9).

It has been demonstrated that the activation of NF- κ B occurs predominantly in the bronchiolar epithelium. In asthma, NF- κ B activation in airway epithelial cells and other cell types (resident macrophages, inflammatory leukocytes, lymphocytes) may affect initiation or maintenance of the inflammatory phenotype that characterizes the disease. In unstimulated cells, NF- κ B is usually kept inactive in the cytoplasm through association with inhibitory proteins of the I κ B (inhibitor of NF- κ B) family. In response to several stimuli, including pro-inflammatory cytokines such as tumor necrosis factor and IL-1, I κ B α is phosphorylated at serines 32 and 36 by the activity of the I κ B kinase complex, ubiquitinated, and degraded by the proteasome, allowing the nuclear translocation of NF- κ B and the transcriptional initiation of NF- κ B-dependent genes. We previously identified ABIN-1 as an intracellular protein that has the potential to inhibit tumor necrosis factor- and IL-1-induced NF- κ B activation upon overexpression (10–12). Because of the potential role of NF- κ B in airway inflammation, it has been proposed that inhibitors of NF- κ B activation may have therapeutic potential in allergic lung inflammation (13, 14). In the present study, we investigated whether adenoviral gene transfer in the lung of the NF- κ B inhibitory protein ABIN-1 could abrogate allergen-induced airway inflammatory responses in a mouse model of asthma.

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¹ The abbreviations used are: Th2, T helper-2; BAL, bronchoalveolar lavage; HRP, horse radish peroxidase; IgE, immunoglobulin E; I κ B, inhibitor of NF- κ B; I κ B α ^s, I κ B α superrepressor; IL, interleukin; IT, intratracheal; NF- κ B, nuclear factor- κ B; OVA, ovalbumin; pfu, plaque forming units; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; E1, ubiquitin-activating enzyme; E3, ubiquitin-protein isopeptide ligase.

MATERIALS AND METHODS

Animals—Eight-week-old female BALB/c mice were purchased from IFFA Credo and were housed under specific pathogen-free conditions in micro-isolator units. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Recombinant Adenovirus Production—The murine ABIN-1 cDNA, N-terminal fused to an E tag, was amplified via PCR with a forward 5'-cgggattccgccatgggtgcgcgggtgcc-3' primer and reverse 5'-ccccaagcttaaatgaccactgcagcc-3' primer. A recombinant adenoviral vector AdABIN-1 was generated by cloning the ABIN-1 PCR fragment into a BamHI and HindIII opened pACpLpA.CMV shuttle vector and co-transfected with the rescue plasmid pJM17 (which encodes the adenovirus dl309 genome, lacking E1 and E3 functions) into HEK293 cells via calcium phosphate coprecipitation. Recombinant plaques were isolated, extracted DNA was verified via PCR, and expression of the correct transgene from the ubiquitously active cytomegalovirus promoter was confirmed by means of Western blotting. Control viruses without transgene (AdRR5) or expressing the β -galactosidase gene (AdLacZ) and a virus expressing the I κ B α superrepressor (AdI κ B α^s) (15) (gift from Dr. R. Hay, University of St. Andrews) were generated with the same pJM17 adenoviral backbone vector. A virus expressing an NF- κ B luciferase reporter gene (AdNF κ BLuc) (16) was obtained from Dr. B. McGray (University of Iowa College of Medicine). High titer virus stocks were prepared in HEK293 cells and purified via single CsCl banding. Titers were determined via plaque assay in HEK293 cells and calculated as plaque forming units (pfu)/ml virus stock.

Analysis of ABIN-1 Expression upon Adenoviral Delivery in Lung Tissue—To demonstrate the expression of ABIN-1 in lung tissue, BALB/c mice were anesthetized by intraperitoneal injection with 200 μ l of a mixture of xylazine (10 μ g/g) and ketamine (100 μ g/g) and placed in a supine position at a head-up angle of $\sim 30^\circ$. 2×10^9 pfu of AdABIN-1 virus in 80 μ l of PBS were administered slowly intratracheally (IT) via the mouth using a floppy tipped gel loading tip attached to a Gilson pipette. Lungs were removed daily, cut in small pieces, and homogenized by douncing in lysis buffer (1% Nonidet P-40, 200 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10% glycerol) supplemented with 0.1 mM aprotinin and leupeptin. After 20 min of incubation on ice, homogenates were centrifuged for 15 min at high speed at 4 $^\circ$ C. Protein concentration was determined by Bradford assay (Bio-Rad). 50 μ g of proteins were subjected to SDS-PAGE and immunoblotted with anti-E tag coupled to horse radish peroxidase (HRP). Signals were revealed by ECL (Amersham Biosciences).

For immunohistochemistry, lungs were removed 2 days after adenovirus administration, fixed in 4% paraformaldehyde, and embedded in paraffin. 5- μ m sections were subjected to immunostaining using the anti-E tag HRP antibody.

Sensitization, Treatment, and Challenge Protocol—Five- to six-week-old female BALB/c mice were sensitized on days 0, 7, and 14 by three intraperitoneal injections of 10 μ g of ovalbumin (OVA) adsorbed to 1 mg of Al(OH)₃ (alum). Negative control mice were injected with PBS. At day 20, anesthetized mice were treated via IT instillation with recombinant adenoviruses or PBS as described above. Mice were challenged with two IT injections of 80 μ l of OVA solution (20 μ g/mouse) at days 21 and 22. Bronchoalveolar lavage (BAL), lung removal, and serum collection were performed 24 h after the last challenge.

Bronchoalveolar Lavage—BAL was performed under anesthesia with an intraperitoneal injection of avertin (2.5% in PBS-low endotoxin). A 23-gauge cannula was installed into the trachea, and cells were collected by washing the airway lumen with 2×0.5 ml of PBS. After centrifugation, supernatants were stored at -20° C for cytokine measurement by ELISA. Differential cell counts were determined on cytospin preparations stained with May-Grünwald-Giemsa (Sigma) by classification of 200 cells on standard morphology criteria.

Lung Histology—Lungs were fixed with 4% paraformaldehyde and embedded in paraffin. 5- μ m sections were stained with hematoxylin and eosin for histological analysis.

Measurement of NF- κ B Activity in the Lung—To measure the effect of adenoviral-mediated gene expression of ABIN-1 or I κ B α^s on NF- κ B activity in the lungs, mice were IT-co-injected 24 h before the first OVA challenge with 10^9 pfu AdRR5, AdABIN-1, or AdI κ B α^s and 3×10^8 pfu of a recombinant adenovirus expressing an NF- κ B-dependent luciferase reporter gene (AdNF κ BLuc), as well as 3×10^8 pfu of a virus expressing a cytomegalovirus promoter-driven β -galactosidase transgene (AdLacZ). Expression of β -galactosidase was used to make sure that infection efficiency was similar in all mice. Lungs were removed 24 h after the last OVA challenge and homogenized by douncing in luciferase lysis

buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-cyclohexanediaminetetraacetic acid, 10% glycerol, and 1% Triton X-100). Equal amounts of protein were further diluted with luciferase substrate buffer to a final concentration of 470 μ M luciferin, 270 μ M coenzyme A, and 530 μ M ATP. Luciferase activity was measured in a Topcount microplate scintillation reader (Packard Instrument Co., Meriden, CT). β -Galactosidase activity was assayed using the Galactostar reporter gene assay system (Applied Biosystems). Results are expressed as relative light units/ μ g of protein.

ELISA for OVA-specific IgE, Eotaxin, IL-1 β , and IL-4—OVA-specific IgE, eotaxin, IL-1 β , and IL-4 levels were determined by ELISA. In the case of OVA-specific IgE, ELISA plates (Nunc 96-well immunoplates) were prepared by coating the surface overnight at 4 $^\circ$ C with 50 μ l of OVA (100 μ g/ml) in carbonate buffer and blocking nonspecific binding activity with 10% fetal calf serum in PBS. Diluted samples were incubated in each well for 2 h. Bound IgE was detected with sheep anti-mouse IgE antibody. Secondary anti-sheep IgG antibody coupled to HRP was added, the bound HRP enzyme was detected with tetramethylbenzidine, and the absorbance was read at 450 nm. Values of IgE levels were determined using a standard curve established on the basis of a serial dilution of supernatant of an OVA-specific IgE hybridoma. Arbitrary units were used according to the OD₅₀ of the standard curve. In the case of IL-4, plates were coated with anti-IL-4 monoclonal antibody (18191D; BD Biosciences) in carbonate buffer overnight at 4 $^\circ$ C and blocked with 1% bovine serum albumin in PBS. Diluted samples were incubated in each well overnight at 4 $^\circ$ C. A standard curve was made using recombinant IL-4. After washing the plates, biotinylated anti-IL-4 monoclonal antibody was added and incubated for 1 h at room temperature. Plates were treated with HRP-conjugated streptavidine at room temperature for 1 h and then incubated with peroxidase substrate (tetramethylbenzidine) by standard procedures. The absorbance was measured at 450 nm. Eotaxin and IL-1 β levels in BAL fluid were determined via ELISA according to the manufacturer's instructions (Quantin; R&D Systems Inc.).

RESULTS

Intratracheal Instillation of a Recombinant Adenovirus Results in Transgene Expression in the Airway Epithelium—The accessibility of the airway epithelium makes it an ideal target for viral gene transfer. A replication-deficient adenovirus expressing E-tagged murine ABIN-1 under the control of a cytomegalovirus promoter (AdABIN-1) was used to determine the timing and localization of transgene expression after IT administration. BALB/c mice were infected with 2×10^9 pfu of AdABIN-1 virus diluted in 100 μ l of PBS. ABIN-1 expression in lung tissue at several time points after injection was determined by Western blotting with anti-E tag antibodies. The expression of ABIN-1 was high at day 1 after IT administration and then slowly declined over the next days, becoming almost undetectable at day 6 (Fig. 1A). To determine the cellular localization of ABIN-1 transgene expression in the lungs, we performed separate experiments in which mice were treated with 2×10^9 pfu of AdABIN-1. Two days after IT administration of AdABIN-1, E-tagged ABIN-1 expression was analyzed by immunohistochemistry on paraffin-embedded lung sections. Mice treated with AdABIN-1 showed staining almost exclusively in alveolar and bronchiolar epithelia (Fig. 1B). Staining was not detectable on similarly prepared lung sections from PBS-treated controls. These findings show that adenoviral gene transfer can be used to deliver an ABIN-1 transgene selectively to airway epithelium. Similar results were obtained in the case of other transgenes, including a transgene encoding an I κ B α superrepressor (I κ B α^s) protein (data not shown).

Adenoviral Expression of ABIN-1 Reduces Allergen-induced Eosinophil Infiltration into the Lung—To analyze whether adenoviral expression of the NF- κ B inhibitory protein ABIN-1 in the lung would affect asthmatic inflammation, we made use of a model of OVA-induced allergic airway inflammation. In this model, BALB/c mice were sensitized by three intraperitoneal injections (at days 0, 7, and 14) of 10 μ g of OVA adsorbed to 1 mg of Al(OH)₃ (alum). As a negative control, mice were injected with PBS. At days 21 and 22, mice were challenged with two IT

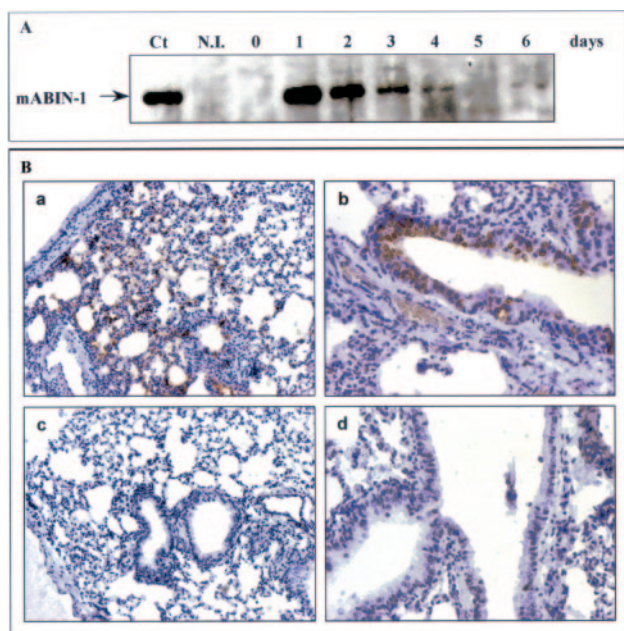


FIG. 1. Lung expression of ABIN-1 upon AdABIN-1 treatment. A, mice were treated IT at day 0 with AdABIN-1, and lungs were removed daily from day 0 to day 6. Lung homogenates were subjected to SDS-PAGE, and E-tagged ABIN-1 expression was revealed by Western blotting using an anti-E tag antibody coupled to HRP. *N.I.*, non-infected mouse used as negative control. *Ct*, positive control: lysate from HEK293T cells transfected with an ABIN-1 expression vector. B, 2 days after IT virus administration, lungs were removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections were immunostained using anti-E tag HRP antibody. Brown precipitates reveal the presence of ABIN-1 in alveolar (a, $\times 100$) and bronchiolar epithelia (b, $\times 200$). Lung sections from non-infected mice and immunostained with anti-E tag HRP antibody were used as negative control (c, $\times 100$, and d, $\times 200$). Results are from a representative experiment.

injections of 80 μ l of OVA solution (20 μ g/mouse). It has previously been shown that OVA challenge results in a rapid and protracted activation of NF- κ B and the production of several NF- κ B-dependent gene products, which corresponded temporally with an influx of eosinophils into the airspaces (17). To investigate the effect of NF- κ B inhibition by ABIN-1 on inflammatory cell infiltration, a recombinant adenovirus expressing E-tagged murine ABIN-1 (AdABIN-1) was delivered via IT instillation to OVA-sensitized mice 1 day before the first OVA challenge. Injection with PBS or an adenovirus expressing no transgene (AdRR5) was used as negative control. A virus that expresses an I κ B α superrepressor protein (AdI κ B α^s) was used as an alternative approach to inhibit NF- κ B activation. The I κ B α^s corresponds to a mutant of I κ B α in which Ser-32 and Ser-36 have been mutated to Ala, preventing its stimulus-induced phosphorylation and degradation and leading to the inhibition of NF- κ B activation (18). To demonstrate the inhibition of NF- κ B-dependent gene transcription in the lung by ABIN-1 or I κ B α^s overexpression, we co-injected a recombinant adenovirus expressing an NF- κ B-dependent luciferase reporter gene (AdNF κ BLuc) as well as a virus expressing a β -galactosidase transgene behind a constitutively active cytomegalovirus promoter (AdLacZ) and measured the amount of luciferase and β -galactosidase activity that was present in lung homogenates 24 h after OVA challenge as a readout for NF- κ B activity. β -Galactosidase activity was similar in all mice, confirming equal infection efficiency. Luciferase activity was elevated 6-fold in lung homogenates of mice that were sensitized and challenged with OVA (OVA/OVA) as compared with non-sensitized mice (PBS/OVA) (Fig. 2), reflecting a strong activation of NF- κ B in the lungs of asthmatic mice. However, OVA/OVA

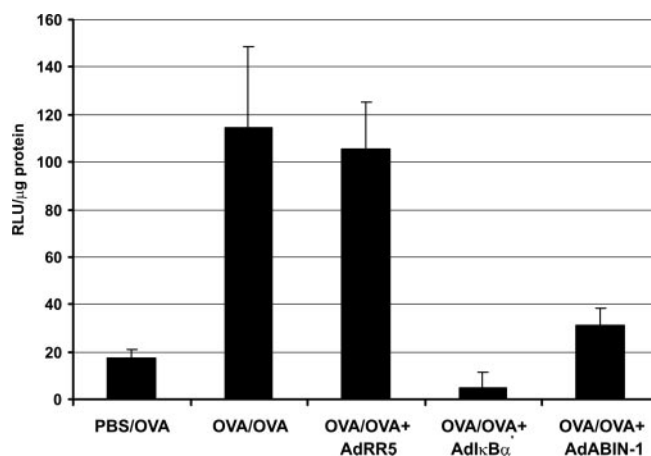


FIG. 2. NF- κ B-dependent luciferase reporter activity in lungs from allergen-sensitized mice. Mice were injected IT with AdNF κ BLuc and AdLacZ 1 day before the first OVA challenge. Lung homogenates were prepared 24 h after the last OVA challenge and assessed for luciferase and β -galactosidase reporter gene activity, which was normalized to total protein. Results are expressed as relative light units/ μ g of total protein (RLU/ μ g). *PBS/OVA*, mice sensitized with PBS and challenged with OVA; *OVA/OVA*, mice sensitized and challenged with OVA; *AdRR5*, *AdI κ B α^s* , and *AdABIN-1*, mice sensitized and challenged with OVA but treated with recombinant adenoviruses AdRR5, AdI κ B α^s , and AdABIN-1, respectively. Results from a representative experiment with 5 mice/group are given as mean $\pm$ S.E.

mice co-infected with AdI κ B α^s or AdABIN-1 showed a strong decrease in luciferase activity, whereas mice infected with a negative control adenovirus (AdRR5) did not. These data clearly demonstrate that adenoviral gene expression of ABIN-1 or I κ B α^s is a powerful tool to inhibit NF- κ B-dependent gene expression in the epithelial airway cells of allergic mice. To analyze the effect of NF- κ B inhibition by ABIN-1 or I κ B α^s expression on inflammatory cell infiltration in the lungs, BAL fluid was collected 24 h after the last OVA challenge and total cell number, as well as differential cell counts for macrophages, neutrophils, and eosinophils, were determined. Mice that were sensitized and challenged with OVA (OVA/OVA) showed a marked influx of cells into the airways, evidenced by increases in cells recovered from the BAL (Fig. 3). Typical of antigen-induced airway responses, the number of cells recovered from BAL was accounted for in large part by the influx of eosinophils. In addition, the number of infiltrating macrophages was increased. A similar cell infiltration pattern was observed in OVA/OVA mice treated with AdRR5, but these mice showed an additional slight increase in the number of neutrophils and lymphocytes, which is in agreement with the reported inflammatory response in the lung that can be induced by high doses of adenoviral vectors (19). In contrast, OVA/OVA mice treated with AdABIN-1 or AdI κ B α^s showed a strong reduction in the number of infiltrating eosinophils.

Concomitant with BAL fluid analysis, airway inflammation was evaluated by histological examination of hematoxylin- and eosin-stained lung tissue sections. Sham-sensitized mice did not develop pulmonary inflammation upon OVA challenge (Fig. 4, A and B). Conversely, widespread perivascular and peribronchiolar inflammatory infiltrates were observed in OVA-sensitized mice after antigen provocation (Fig. 4, C and D). Lung-infiltrating cells were mostly eosinophils. Treatment of OVA-sensitized and -challenged mice with AdABIN-1 and AdI κ B α^s resulted in a significant reduction of eosinophil lung infiltration (Fig. 4, G–J), whereas administration of AdRR5 had no detectable effect (Fig. 4, E and F). These findings show that treatment with adenoviruses expressing NF- κ B inhibitory proteins strongly attenuates allergic airway inflammation.

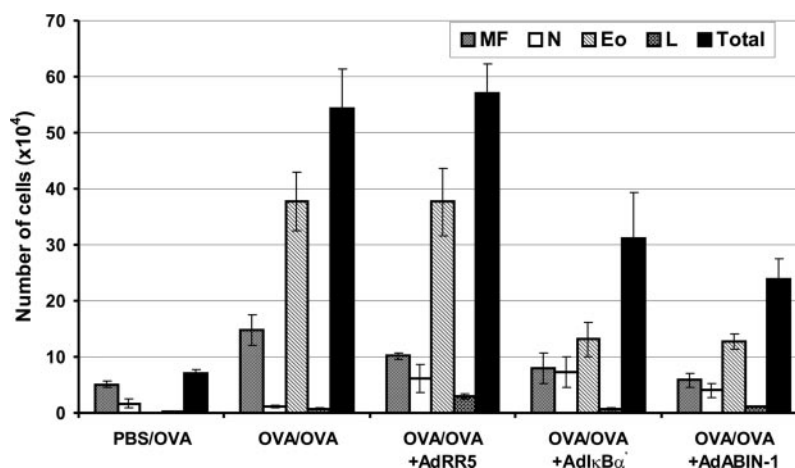


FIG. 3. **Assessment of total cell number and differential cell counts in BAL fluid of allergen-sensitized mice.** BAL fluid was collected 24 h after the last OVA challenge. Total cell number, as well as differential cell counts for macrophages (MF), neutrophils (N), lymphocytes (L), and eosinophils (Eo), are plotted for each group. PBS/OVA, mice sensitized with PBS and challenged with OVA; OVA/OVA, mice sensitized and challenged with OVA; AdRR5, AdIkBa, and AdABIN-1, mice sensitized and challenged with OVA but treated with recombinant adenoviruses AdRR5, AdIkBa, and AdABIN-1, respectively. Results from a representative experiment with 5 mice/group are given as mean $\pm$ S.E.

Adenovirus-mediated Overexpression of ABIN-1 in the Lung Reduces Eotaxin, IL-4, and IL-1 β Levels in Allergen-exposed Airways—Eotaxin is a chemokine that is essential for the recruitment and activation of eosinophils into the airways and potentially also for the development of pulmonary fibrosis as a result of sensitization and challenge with allergen (20). Moreover, NF- κ B, in cooperation with STAT-6, which is activated in response to Th2 cytokines IL-4, IL-5, and IL-13, is believed to be responsible for enhanced eotaxin expression by airway epithelial cells in asthma. We therefore determined the levels of eotaxin in BAL fluid from OVA-sensitized and -challenged mice. As expected in this model, eotaxin levels in BAL fluid from OVA/OVA mice were increased 4-fold compared with PBS/OVA control mice (Fig. 5A). However, eotaxin concentrations in the BAL fluid of AdABIN-1-infected OVA/OVA mice showed a >50% reduction as compared with the OVA/OVA mice that were treated with PBS or the AdRR5 control virus.

We next measured the concentration of IL-4 and IL-1 β in the BAL fluid recovered from the animals. The IL-4 signaling pathway controls the most important cellular developmental events that underlie asthma. These include Th2 cell activation, B cell activation and IgE secretion, mast cell development, and effector events related exclusively to immune effects on the lung such as goblet cell metaplasia and airway hyperresponsiveness (21). The pro-inflammatory cytokine IL-1 β has been shown to be required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response (22). As expected, BAL fluid levels of IL-4 and IL-1 β were markedly increased in OVA/OVA mice compared with PBS/OVA controls (Fig. 5, B and C). Mice treated with AdABIN-1 showed an almost complete suppression of IL-4 and IL-1 β production. These data show that IT delivery of adenoviruses expressing the NF- κ B inhibitory protein ABIN-1 significantly reduces IL-4 and IL-1 β production in the lungs of OVA/OVA mice, which is consistent with a reduction of eosinophilia and eotaxin production in AdABIN-1-infected mice.

Overexpression of ABIN-1 in Airway Epithelia Reduces OVA-specific IgE Levels in the Serum of Allergen-treated Mice—IgE and mast cells are believed to play important roles in allergic inflammation. IgE can capture the antigen presented to the airways, and the immune complexes so formed can augment allergic airway responses in a high affinity IgE receptor-dependent manner. Levels of OVA-specific IgE in mouse sera, collected 24 h after the last OVA challenge, were measured by ELISA. OVA sensitization and challenge induced a 1000-fold

increase in serum IgE levels (Fig. 5D). However, a 10–20-fold decrease in IgE levels could be seen in sera from OVA/OVA mice treated with AdABIN-1 compared with OVA/OVA mice treated with AdRR5 control virus. These results indicate that ABIN-1 overexpression in airway epithelia significantly reduces specific IgE production in allergen airway inflammation.

DISCUSSION

Using a murine model of allergen-induced asthma, we have shown that a single IT administration of an adenovirus expressing the NF- κ B inhibitory protein ABIN-1 effectively suppresses allergic inflammation in the lungs. IT delivery of recombinant adenoviral vectors has previously been shown to target mainly airway epithelium (23), which is also reflected by the high expression of ABIN-1 in alveolar and bronchiolar epithelium that we could observe in the present study. The airway epithelium represents an important site of interaction with allergens. These induce various signaling events that lead to the production of mediators that contribute to the generation or perpetuation of inflammation and that may facilitate the development of asthma (24). In the present study, NF- κ B inhibition in airway epithelial cells by overexpression of ABIN-1 was shown to reduce allergen-induced eosinophil infiltration, a hallmark of asthma. This is in line with our finding that ABIN-1 also reduced the levels of eotaxin in the BAL fluid. It was reported that this chemokine is highly expressed by lung epithelial cells in asthmatic patients and correlates with eosinophil infiltration in lung (3, 25, 26). The presence of an NF- κ B binding site in the eotaxin promoter (27) suggests that ABIN-1 directly affects the NF- κ B-dependent transcription of the eotaxin gene. We also showed that adenoviral transfer of ABIN-1 decreases the levels of IL-1 β in the lungs of OVA-sensitized mice. IL-1 β is a pro-inflammatory cytokine that is produced by airway epithelial cells and several immune cells. This cytokine is of particular interest in asthma because it can lead to the NF- κ B-dependent expression of eotaxin and eosinophil recruitment after allergen challenge (28, 29). Moreover, IL-1 β is involved in macrophage maturation and helps the presentation of antigen (allergen) to T helper cells, leading to the development of a Th2 profile. Inhibition of IL-1 β production could therefore result in a decrease of Th2 cells and consequently lead to the reduction of IL-4 and other Th2 cytokines. Indeed, IL-4 levels were significantly reduced in BAL fluid of OVA/OVA mice treated with AdABIN-1. Although, it has recently been shown that NF- κ B is directly involved in IL-4

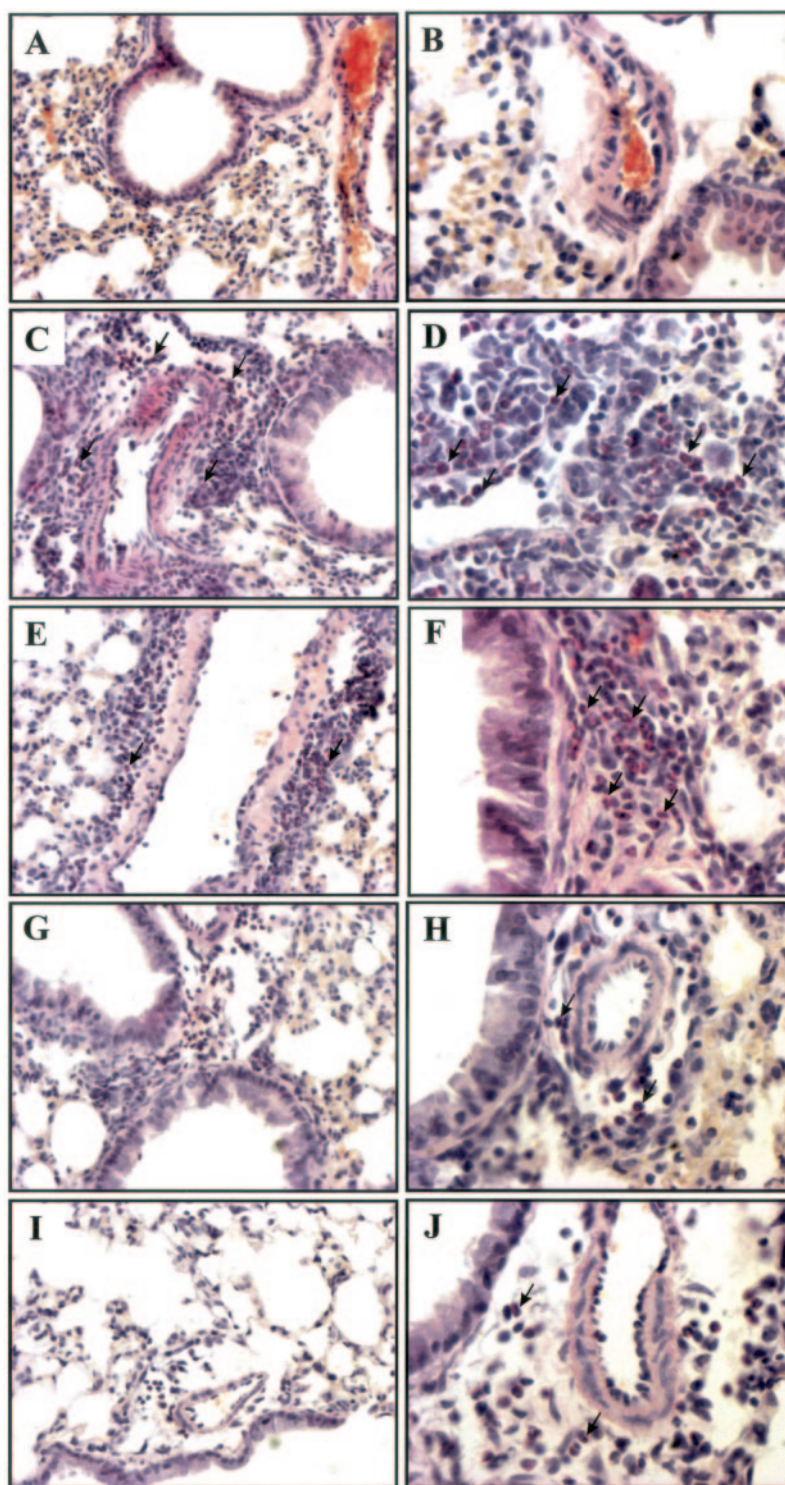


FIG. 4. Histological evaluation of lung inflammation following OVA sensitization and treatment with different recombinant adenoviruses. Paraffin-embedded lung sections were prepared 24 h after the last OVA challenge and stained with hematoxylin and eosin. *A* and *B*, mice sensitized with PBS and challenged with OVA = PBS/OVA ($\times 200$ and $\times 400$). *C* and *D*, mice sensitized and challenged with OVA = OVA/OVA ($\times 200$ and $\times 400$). *E* and *F*, OVA/OVA mice treated with AdRR5 ($\times 200$ and $\times 400$). *G* and *H*, OVA/OVA mice treated with AdIkB* ($\times 200$ and $\times 400$). *I* and *J*, OVA/OVA mice treated with AdABIN-1 ($\times 200$ and $\times 400$). Arrows indicate eosinophil (pink cytoplasm) infiltration. Results are from a representative experiment.

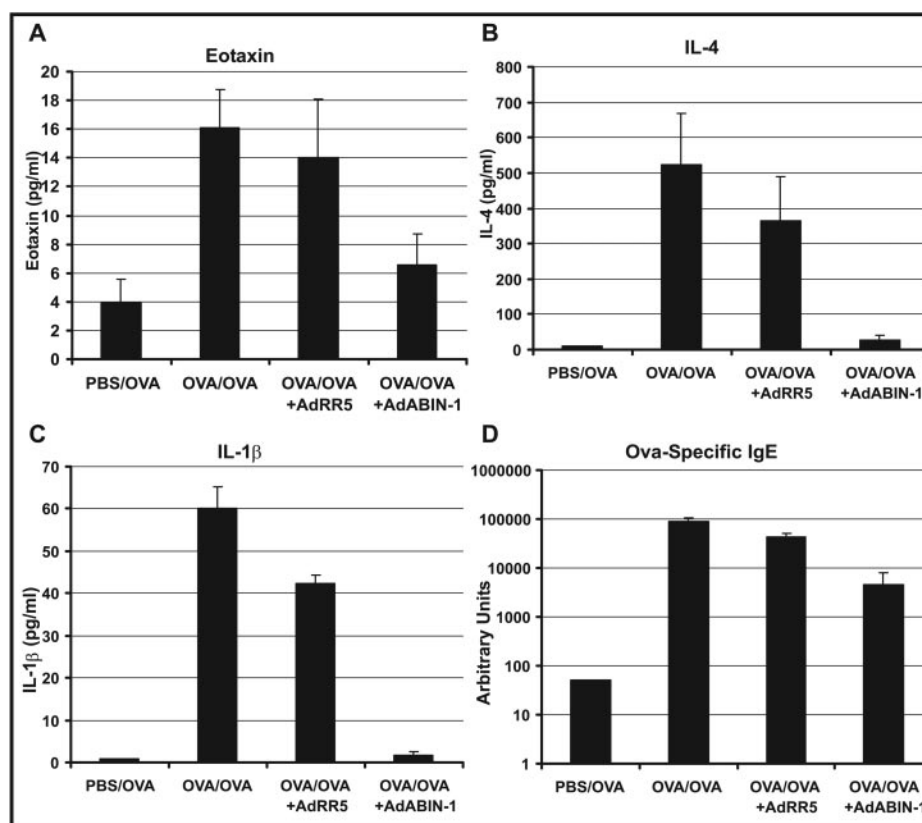
transcription in activated T-cells, a direct effect of ABIN-1 on IL-4 transcription is unlikely in view of the low efficiency of adenoviruses to infect T cells. The decreased IL-4 levels most likely reflect the inhibitory effect of the NF- κ B inhibitory proteins on IL-1 β production, thus resulting in a decrease of Th2 cells. IL-4 is known to inhibit eosinophil apoptosis and promote eosinophilic inflammation by inducing eosinophil chemotaxis and activation through the increased expression of eotaxin (30, 31). Moreover, IL-4 also induces the production of IgE by B lymphocytes (32).

To the best of our knowledge, this is the first demonstration of direct inhibitors of the NF- κ B signaling pathway interfering

with allergic airway inflammation. In the case of ABIN-1, the molecular mechanism of NF- κ B inhibition is still unknown, but there is considerable evidence that ABIN-1 inhibits NF- κ B activation upstream of the I κ B kinase complex (11),² which is believed to be the point of convergence for many stimulus-specific NF- κ B activation pathways. In contrast, the I κ B α^s protein, which is also used in the present study, interferes downstream of I κ B kinase. Therefore, the NF- κ B inhibitory effect of ABIN-1 might be more stimulus-specific than the effect

² K. Heyninck, unpublished observations.

FIG. 5. Assessment of pro-inflammatory gene expression in BAL fluid and OVA-specific IgE levels in serum of allergen-sensitized mice treated with AdABIN-1. Eotaxin, IL-1 β , and IL-4 levels in BAL fluid, as well as serum IgE from OVA-sensitized and -challenged mice, were measured 24 h after the last OVA challenge and determined by specific ELISA. A, eotaxin levels in BAL fluid. B, IL-4 levels in BAL fluid. C, IL-1 β levels in BAL fluid. D, OVA-specific IgE in serum. Results from a representative experiment with 5 mice/group are given as mean $\pm$ S.E.



of I κ B α ^s. Measurement of the NF- κ B activity in lung homogenates of asthmatic mice by means of an NF- κ B-dependent luciferase reporter assay revealed that the general NF- κ B inhibitory protein I κ B α ^s was slightly more potent than ABIN-1 for inhibiting NF- κ B activation. Although the identity of the stimuli that are responsible for the increased NF- κ B activity observed in asthmatic mice is still unclear, the above observation might reflect a more stimulus-specific effect of ABIN-1. Nevertheless, the anti-inflammatory effect of ABIN-1 was in most of our experiments as strong as the effect obtained with I κ B α ^s, indicating that ABIN-1 lowers NF- κ B activity to a level that no longer elicits an inflammatory response. Because NF- κ B is a redox-sensitive transcription factor, the inhibitory effect of several antioxidants in the OVA-induced asthma model has also been suggested to reflect their inhibitory effect on NF- κ B (33). However, such agents can be expected to affect several other redox-sensitive molecules apart from NF- κ B. Inhaled glucocorticoids, which inhibit NF- κ B- and AP-1-dependent transcription, are also important anti-inflammatory agents in asthma management. However, glucocorticoids, particularly at high doses, have significant and severe adverse effects (34). In addition, a group of severe asthmatic patients had a poor response to glucocorticoid treatment and may be glucocorticoid-resistant (35). While this paper was under preparation, a report describing the IT administration of “naked” NF- κ B decoy oligodeoxynucleotides to OVA-sensitized mice was published (36). Interestingly, this strategy led to efficient transfection of airway immune cells, but not constitutive lung cells, which is in contrast to the adenoviral gene transfer method used in our study, which mainly transduces lung epithelial cells. Although previous studies demonstrated increased NF- κ B activity in bronchial epithelium and airway immune cells of OVA-sensitized mice upon allergen challenge (17, 37), the specific functions of the NF- κ B pathway in various lung cell types are not well defined. The role of several immune cells in the development of allergic airway inflammation is well accepted, but it

has been demonstrated that airway epithelial cells can also be stimulated to activate NF- κ B and produce cytokines and chemokines that are important for directing airway inflammation (23). Taken together, our findings and the studies of Desmet et al. (36) demonstrate that activation of NF- κ B in local immune cells as well as lung epithelial cells is critically involved in allergic airway disease and that targeting NF- κ B in either immune cells or airway epithelial cells is sufficient to attenuate an inflammatory response in the lung. It should also be noted that, although NF- κ B inhibition by NF- κ B decoys was associated with strong attenuation of allergic lung inflammation and local production of IL-13 and eotaxin, in contrast to our studies, IL-4 and OVA-specific IgE production was not reduced. The reason for this different outcome is still unclear but likely reflects the difference in cells that are targeted by the administration of NF- κ B decoy oligonucleotides or adenoviruses, respectively.

In summary, we have shown that overexpression of the NF- κ B inhibitory protein ABIN-1 inhibits an allergic inflammatory response in the lung that is correlated with decreased eosinophilia and strongly reduced expression of eotaxin, IL-1 β , and IL-4, as well as serum IgE levels. These findings not only prove that NF- κ B plays a critical role in the pathogenesis of allergic inflammation but also illustrate that inhibiting NF- κ B could have therapeutic value in the treatment of asthma and other chronic inflammatory lung diseases. Although the present study demonstrates that gene transfer of NF- κ B inhibitory proteins offers the opportunity to inhibit an allergic respiratory response, numerous hurdles remain before therapeutic gene transfer of asthma can be considered. However, the evolution of better gene transfer vehicles causes optimism that gene therapy may some day replace other medications.

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