

Selective Blockade of NF- κ B Activity in Airway Immune Cells Inhibits the Effector Phase of Experimental Asthma¹

Christophe Desmet,* Philippe Gosset,[‡] Bernard Pajak,[§] Didier Cataldo,[†] Mohamed Bentires-Alj,[‡] Pierre Lekeux,* and Fabrice Bureau^{2*}

Knockout mice studies have revealed that NF- κ B plays a critical role in Th2 cell differentiation and is therefore required for induction of allergic airway inflammation. However, the questions of whether NF- κ B also plays a role in the effector phase of airway allergy and whether inhibiting NF- κ B could have therapeutic value in the treatment of established asthma remain unanswered. To address these issues, we have assessed in OVA-sensitized wild-type mice the effects of selectively antagonizing NF- κ B activity in the lungs during OVA challenge. Intratracheal administration of NF- κ B decoy oligodeoxynucleotides to OVA-sensitized mice led to efficient nuclear transfection of airway immune cells, but not constitutive lung cells and draining lymph node cells, associated with abrogation of NF- κ B activity in the airways upon OVA provocation. NF- κ B inhibition was associated with strong attenuation of allergic lung inflammation, airway hyperresponsiveness, and local production of mucus, IL-5, IL-13, and eotaxin. IL-4 and OVA-specific IgE and IgG1 production was not reduced. This study demonstrates for the first time that activation of NF- κ B in local immune cells is critically involved in the effector phase of allergic airway disease and that specific NF- κ B inhibition in the lungs has therapeutic potential in the control of pulmonary allergy. *The Journal of Immunology*, 2004, 173: 5766–5775.

The worldwide prevalence and severity of allergic asthma have increased alarmingly over the last decades (1). The pathophysiological features of allergic asthma, chronic pulmonary eosinophilia, airway hyperresponsiveness (AHR)³ to a variety of nonspecific spasmogenic stimuli, excessive airway mucus production, and elevated serum IgE levels, have all been linked to aberrant Th2 cell responses to commonly inhaled allergens (see review Refs. 2–5). Allergen-specific Th2 cells exert their critical effector functions by producing a unique repertoire of cytokines, the most important of which being IL-4, IL-5, and IL-13 (2–5).

NF- κ B is a pleiotropic transcription factor that plays an important role in regulating the expression of multiple genes involved in immune responses (see review Ref. 6). The NF- κ B family is composed of five structurally related DNA-binding proteins, designated NF- κ B1/p50, NF- κ B2/p52, RelA/p65, Rel/c-Rel, and RelB. Although the most common NF- κ B complex is a heterodimer of the p65 and p50 subunits, the different family members can associate in various homo- or heterodimer combinations. Inactive

NF- κ B complexes are sequestered in the cytosol by inhibitory proteins of the I κ B family. Following various stimuli, I κ B proteins are phosphorylated, ubiquitinated and degraded, allowing NF- κ B nuclear translocation and transcriptional initiation of NF- κ B-dependent genes.

It has been demonstrated in humans and animal models that asthmatic inflammation is associated with increased NF- κ B activity in lung cells (7–9). To further investigate the role of NF- κ B in asthma, p50- and c-Rel-deficient mice were tested in a model of OVA-induced allergic airway inflammation (9–11). These studies showed that p50^{−/−} mice are incapable of mounting eosinophilic airway inflammation due to the inability of their naive T cells to differentiate along the Th2 lineage (9). Indeed, p50 is required for Gata-3 expression (10), a key step in Th2 differentiation and Th2 cytokine expression (12, 13). Similarly, defects in T cell activation and proliferation preclude induction of allergic airway inflammation in c-Rel^{−/−} mice (11).

Although studies of p50 and c-Rel knockout mice revealed an essential role for NF- κ B in induction of allergic airway inflammation, the questions of whether NF- κ B also plays a role in the effector phase of pulmonary allergy and whether inhibiting NF- κ B function can be used for the therapy of established asthma remain unanswered. To address these issues, we have assessed in OVA-sensitized wild-type mice the effects of selectively antagonizing NF- κ B activity in the lungs during OVA provocation. Specific NF- κ B inhibition was achieved using NF- κ B decoys. Decoys are synthetic double-stranded oligodeoxynucleotides (ODNs) bearing the consensus binding sequence of a specific transcription factor (14–16). When introduced into cells, decoys impair the authentic interaction between the target transcription factor and genomic DNA, with subsequent inhibition of gene expression (14–16).

Materials and Methods

NF- κ B decoy ODNs

Double-stranded NF- κ B decoy ODNs containing the consensual NF- κ B binding site (GGGATTTCCC) were generated using equimolar amounts of single-stranded sense and antisense phosphorothioate-modified ODNs (sense strand: 5'-CCT TGA AGG GAT TTC CCT CC-3') as described

Laboratoires de *Physiologie, [†]Service de Pneumologie, and [‡]Chimie Médicale, Centre de Thérapie Cellulaire et Moléculaire, Université de Liège, Liège, Belgium; [§]Institut National de la Santé et de la Recherche Médicale, Unité 416, Institut Pasteur de Lille, Lille, France; and [§]Laboratoire de Physiologie Animale, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, Gosselies, Belgium

Received for publication September 29, 2003. Accepted for publication August 19, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was partly supported by grants from the Union Chimique Belge Pharma, Belgium, and the Ministère de la Région Wallonne, Belgium. C.D. is a research fellow, and F. B., D.C., and M.B.-A. are postdoctoral researchers at the Fond National de la Recherche Scientifique, Belgium. P.G. is a member of the Institut National de la Santé et de la Recherche Médicale, France.

² Address correspondence and reprint requests to Dr. Fabrice Bureau, Laboratoire de Physiologie, Centre de Thérapie Cellulaire et Moléculaire, Université de Liège, Boulevard de Colonster, Bâtiment B42, Sart-Tilman, B-4000 Liège, Belgium. E-mail address: fabrice.bureau@ulg.ac.be

³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; MCh, methacholine; ODN, oligodeoxynucleotide; PAS, periodic acid-Schiff; Penh, enhanced pause; TLN, thoracic lymph node.

(17). Briefly, synthetic single-stranded ODNs were dissolved in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), purified by HPLC and quantified by spectrophotometry (Eurogentec, Liège, Belgium). Single-stranded ODNs were then annealed for 2 h, during which time the temperature was reduced from 80°C to 25°C. Double-stranded scrambled ODNs were used as controls (sense strand: 5'-TTG CCG TAC CTG ACT TAG CC-3'). In some experiments, sense strand of NF- κ B decoys and scrambled ODNs was FITC-labeled or biotinylated at both 5' and 3' ends.

Induction of allergic airway disease and treatment with NF- κ B decoys

Six- to 8-wk-old female BALB/c mice (Harlan Nederland, Horst, The Netherlands) were sensitized on days 0 and 14 by i.p. injection of 10 μ g of OVA (grade III; Sigma-Aldrich, St. Louis, MO) dissolved in 100 μ l of PBS and mixed with 100 μ l of Inject Alum (Pierce, Rockford, IL). Sham-immunized mice received alum alone. On days 28, 29, and 30, mice were challenged by exposure to an aerosol of 1% (wt/v) OVA in PBS for 1 h. Inhalation was conducted in a plastic chamber connected to the aerosol output from a Ultra-Neb99 nebulizer (DeVilbiss, Somerset, PA). Twenty-four hours after the last challenge (day 31), AHR was measured, the mice were killed, and allergic airway inflammation was characterized.

To assess the effects of specific NF- κ B inhibition on pulmonary allergy, NF- κ B decoys and scrambled ODNs were given by intratracheal instillation (15 nmol in 30 μ l of TE buffer/mouse) on days 28 and 30 (6 h before OVA inhalation) to OVA-sensitized mice. For instillation, mice were anesthetized by i.p. injection of xylazine (200 μ g/mouse) and ketamine (2 mg/mouse). In experiments aimed at localizing NF- κ B decoys or scrambled ODNs, the last intratracheal administration was performed with FITC-labeled or biotinylated ODNs. The protocol was approved by the Animal Ethics Committee of the University of Liège, Belgium.

Measurement of AHR

Responsiveness to β -methacholine (MCh) was assessed in conscious, unrestrained mice by using barometric whole-body plethysmography (Buxco Electronics, Troy, NY) and increases in enhanced pause (Penh) as an index of airway obstruction (18).

Mice were placed in separate plethysmographic chambers and allowed to acclimatize for 10 min before analysis. Baseline readings were taken and averaged for 3 min. Afterward, PBS or increasing concentrations of MCh (1.5 to 12 mg/ml saline) were nebulized into the chambers for 3 min, and Penh measurements were taken and averaged for 3 min after each nebulization. Airway reactivity was expressed as a fold-increase of Penh for each concentration of MCh compared with Penh value after PBS challenge.

Bronchoalveolar lavage, cytology, and cytokine assays

Mice were killed and the lungs and heart were surgically exposed. The trachea was catheterized and the lungs were lavaged with 1 ml of PBS. Cell density in bronchoalveolar lavage fluid (BALF) was assessed by the use of a hemocytometer. Cell differentials were performed on cytospin preparations stained with Diff-Quick (Dade Behring, Duding, Germany). BALF levels of IL-2 (>3 pg/ml; Endogen, Cambridge, MA), IL-4 (>5 pg/ml; Endogen), IL-5 (>5 pg/ml; Endogen), IL-13 (>1.5 pg/ml; R&D Systems, Minneapolis, MN), IFN- γ (>10 pg/ml; Endogen) and eotaxin (>3 pg/ml; R&D Systems) were determined by ELISA.

Lung histology and immunohistochemistry

For routine histology, lungs were fixed in 10% Formalin, paraffin embedded, cut in 5- μ m sections, and stained with H&E. Intracytoplasmic and luminal mucin was assessed by periodic acid-Schiff (PAS) stains.

Biotinylated decoys and biotinylated scrambled ODNs were localized by immunohistochemistry, using the Immunohistowax processing method (A PHASE, Gosselies, Belgium) (19). Double staining was used to identify cell populations that had incorporated biotinylated decoys or biotinylated scrambled ODNs. Briefly, excess biotin from ODNs was blocked using the Vector blocking kit (Vector Laboratories, Burlingame, CA). Slides were then incubated for 1 h at room temperature with biotinylated mAbs directed against I-E^k (14-4-4S; American Type Culture Collection (ATCC), Manassas, VA), F4/80 (A3-1; Serotec, Oxford, U.K.), CD11c (N418; ATCC), B220 (RA3-6B2; BD Pharmingen, San Diego, CA) or Gr-1 (RB6-8C5; BD Pharmingen), or FITC-conjugated Abs directed against CD90/Thy1.2 (53-2.1; BD Pharmingen). Finally, slides were incubated in avidin-biotin-alkaline phosphatase complex (Vectastain ABC kit; Vector Laboratories) and stained with the alkaline phosphatase substrate kit III (Vector Laboratories), or directly stained with anti-FITC alkaline phosphatase Fab (Roche, Mannheim, Germany).

Flow cytometry

To localize FITC-labeled decoys and FITC-labeled scrambled ODNs, dendritic cells, macrophages, eosinophils, and T cell and B cell from BALF, lung tissue, and thoracic lymph nodes (TLNs) were examined for FITC positivity by using flow cytometry. Lungs and TLNs were minced and filtered through nylon mesh before flow cytometry analyses. Cells were incubated (for 30 min on ice in PBS containing 1% normal mouse serum) with either biotin-conjugated anti-mouse CD11c (HL3; BD Pharmingen) and PE-conjugated anti-mouse I-A^d (AMS-32.1; BD Pharmingen) Abs, PE-conjugated F4/80 (CI-A3-1; Serotec), CyChrome-labeled anti-mouse CD3 ϵ (145-2C11; BD Pharmingen), and allophycocyanin-conjugated anti-mouse CD19 (1D3; BD Pharmingen) Abs, PE-conjugated anti-mouse CCR3 Abs (83101; BD Pharmingen), or isotype controls (BD Pharmingen). Biotin labeling was revealed by addition of allophycocyanin-conjugated streptavidin (BD Pharmingen). Cells were washed, fixed with paraformaldehyde (0.25%), and analyzed using a FACSCalibur (BD Biosciences, San Jose, CA).

Apoptosis assays

Dendritic cells, macrophages, eosinophils and T cells from BALF and lung tissue were assayed for apoptosis by staining with Annexin V^{FITC} (Roche). Flow cytometry analyses were performed with a FACSCalibur (BD Biosciences).

Preparation of nuclear extracts from whole lung

Lungs were snap frozen in liquid N₂ and pulverized. The lung powder was homogenized in 5 ml of cytoplasmic extraction buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.2% (v/v) Nonidet P-40, and 1.6 mg/ml protease inhibitors (complete) from Roche in a Dounce tissue homogenizer (Merck Eurolab, Leuven, Belgium) and centrifuged to remove cellular debris. Pelleted nuclei were resuspended in 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.63 M NaCl, 25% (v/v) glycerol, and 1.6 mg/ml protease inhibitors (nuclear buffer), incubated for 20 min at 4°C and centrifuged for 30 min at 14,000 rpm. The same method was used to prepare nuclear extracts from BALF cells. Protein amounts were quantified with the Micro BCA protein assay reagent kit (Pierce).

EMSAs

Binding reactions were performed for 30 min at room temperature with 5 μ g nuclear proteins in 20 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1% (wt/v) acetylated BSA, 3 μ g of poly(dI-dC) (Amersham Pharmacia Biotech, Aylesbury, U.K.), 1 mM DTT, 1 mM PMSF, and 100,000 cpm of ³²P-labeled double-stranded oligonucleotide probes. Probes were prepared by annealing the appropriate single-stranded oligonucleotide (Eurogentec) at 65°C for 10 min in 10 mM Tris, 1 mM EDTA, 10 mM NaCl, followed by slow cooling to room temperature. The probes were then labeled by end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase I (Roche), with [α -³²P]-dATP and [α -³²P]-dCTP (DuPont/NEN Life Science Products, Les Ulis, France). Labeled probes were purified by spin chromatography on Sephadex G-25 columns (Roche). DNA-protein complexes were separated from unbound probe on 4% native polyacrylamide gels at 150 V in 0.25 M Tris, 0.25 M sodium borate, and 0.5 mM EDTA, pH 8.0. Gels were vacuum-dried and exposed to Fuji x-ray film at -80°C for 12 h. To confirm specificity, competition assays were performed with a 50-fold excess of unlabeled wild-type probes and with mutated probes. For supershifting experiments, 1.5 μ l of each Ab was incubated with the extracts for 30 min before addition of the radiolabeled probe. The sequences of the oligonucleotide used in this work were as follows: wild-type palindromic κ B probe, 5'-TTG GCA ACG GCA GGG GAA TTC CCC TCT CCT TAG GTT-3'; mutated palindromic κ B probe, 5'-TTG GCA ACG GCA GAT CTA TTC CCC TCT CCT TAG GTT-3'. The Abs specific for p50 (sc-114), p52 (sc-298), p65 (sc-109), c-Rel (sc-70), and RelB (sc-226) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Determination of serum levels of total and OVA-specific IgG

Serum levels of total IgE and OVA-specific IgE, IgG1, and IgG2a were measured by ELISA. Briefly, 96-well plates (Immulon 2; Dynatech Laboratories, Chantilly, VA) were coated with either purified anti-mouse IgE (5 μ g/ml, 27.74; BD Pharmingen) or OVA (100 μ g/ml). After addition of serum samples, a sheep anti-IgE Ab (affinity purified Abs; Calbiochem, La Jolla, CA) was added to individual wells and its binding was detected with peroxidase-conjugated anti-sheep IgG (affinity purified Abs; Calbiochem). OVA-specific IgG1 and IgG2a were detected using peroxidase-labeled goat anti-IgG1 and anti-IgG2a Abs (affinity purified Abs; Southern Biotechnology Associates, Birmingham, AL). The OVA-specific Ab levels

were calculated as the inverse of the dilution giving 50% of the OD obtained with dilutions of a reference serum pool. Total IgE concentrations were calculated by comparison with commercial mouse IgE standards (BD Pharmingen). The limit of detection for total IgE was 100 μ g/ml.

Restimulation of TLN cells

Cells (3×10^6 in a 24-well plate) isolated from TLNs were restimulated *in vitro* in the presence or absence of 100 μ g/ml OVA. Supernatants were harvested after 4 days and IL-4, IL-5, and IL-13 levels were determined by ELISA.

Statistical analysis

Data are presented as means $\pm$ SDs. The differences between mean values were estimated using either an ANOVA with subsequent Fisher's protected least significant difference tests or a Student's *t* test for unpaired data. A value of *p* < 0.05 was considered significant.

Results

Intratracheal administration of NF- κ B decoy ODNs abrogates NF- κ B activity in the airways upon allergen (OVA) provocation

We first examined in OVA-sensitized mice whether the NF- κ B decoy approach could be applied as an efficient strategy to antagonize NF- κ B activation in the lungs upon OVA provocation. Nuclear extracts prepared from the whole lung or BALF cells of sham-immunized and OVA-challenged mice (hereafter referred to as sham/OVA) demonstrated a weak basal NF- κ B-binding activity, as assessed by EMSAs (Fig. 1*a*, lane 1). As expected, NF- κ B activity was much greater in extracts prepared from the whole lung or BALF cells of mice sensitized and challenged with OVA (OVA/OVA) (Fig. 1*a*, lane 2). Two distinct NF- κ B complexes were found in nuclear extracts from sham/OVA and OVA/OVA mice (Fig. 1*a*, lanes 1 and 2). Supershift assays demonstrated that the slower migrating complex, which was displaced by both anti-p65 and anti-p50 Abs (Fig. 1*b*, lanes 2 and 4), was formed mainly of prototypical p65-p50 heterodimers, whereas the faster migrating complex, which reacted only with anti-p50 Abs (Fig. 1*b*, lane 4), corresponded to p50 homodimers. The NF- κ B complexes were not supershifted by the Abs directed against p52, c-Rel, and RelB (Fig. 1*b*, lanes 3, 5, and 6). As shown in Fig. 1*a*, intratracheal delivery of NF- κ B decoys markedly inhibited activity of both NF- κ B complexes in the airways of OVA/OVA mice (Fig. 1*a*, lane 4), whereas control scrambled ODNs had no effects (Fig. 1*a*, lane 3). Oct-1-binding activity, used as a loading control, was comparable in all samples (Fig. 1*a*, lanes 1–4). DNA-binding competition experiments using 50-fold excess of unlabeled wild-type and mutated palindromic κ B probes confirmed specificity of NF- κ B binding (data not shown). Taken together, these data show that local administration of decoy ODNs efficiently antagonizes NF- κ B activation in the whole lung and BALF cells of OVA-sensitized mice following OVA challenge.

Decoy ODNs inhibit NF- κ B activation in the airways of OVA-challenged mice by targeting local immune cells

To localize NF- κ B decoys *in vivo*, the last intratracheal decoy administration (6 h before the last OVA challenge) was performed with FITC-labeled ODNs. At 24 h later, cells from BALF, lung tissue, and TLNs were examined for FITC positivity by flow cytometry. Cells from OVA/OVA mice that received unlabeled decoys were used as controls. As shown in Fig. 2, most macrophages (F4/80⁺), dendritic cells (I-A^{high}CD11c^{medium-high}), T cells (CD3e⁺), and eosinophils (large (medium to high forward scatter levels) and granular (high side scatter levels) CCR3⁺ cells were considered as eosinophils; this population indeed contained >90% eosinophils, as determined by flow cytometry sorting and May-Grünwald Giemsa staining) from BALF and lung tissue were FITC⁺. Only rare B cells (CD19⁺) were found in the lungs, but

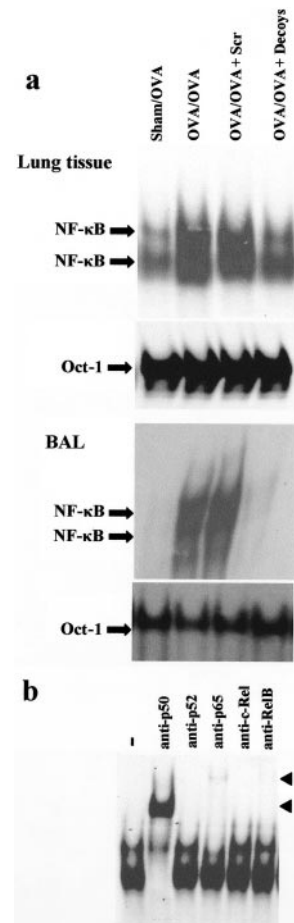


FIGURE 1. Local administration of decoy ODNs suppresses NF- κ B activation in the airways of OVA-challenged mice. Mice were either sensitized with OVA + alum (days 0 and 14) and challenged by repeated exposure to an aerosol of OVA (days 28–30) (OVA/OVA mice) or sham-sensitized with alum only and OVA-challenged (sham/OVA mice). NF- κ B decoys or scrambled ODNs (Scr) were given by intratracheal instillation on days 28 and 30 (6 h before OVA inhalation) to OVA/OVA mice. *a*, Following 24 h after the last OVA challenge (day 31), nuclear protein extracts were prepared from the whole lung or from BALF cells. These extracts were assessed for NF- κ B and Oct-1 DNA-binding activity by EMSAs. *b*, To characterize specific NF- κ B complexes, supershift analysis was conducted with Abs directed against p50, p52, p65, c-Rel, and RelB. Supershifts (solid arrowheads) are indicated. These results are representative of at least 12 comparable experiments.

nearly all had incorporated NF- κ B decoys (data not shown). Cells from TLNs, namely macrophages, dendritic cells, and T and B cells, were all FITC⁺ (Fig. 2).

In vivo distribution of NF- κ B decoys was also evaluated by immunohistochemistry following intratracheal delivery of biotinylated ODNs. Biotinylated NF- κ B decoys 1) targeted peribronchial and perivascular immune cells and 2) were mainly located in the nucleus of these cells (Fig. 3*a*). No signal was detectable in constitutive lung cells, including bronchial epithelial cells, fibroblasts and endothelial cells (Fig. 3*a*). Double staining showed that the majority of the signal was located in the nucleus of peribronchial and perivascular dendritic cells (I-E^{k+} and CD11c⁺), macrophages (F4/80⁺), granulocytes (Gr-1⁺), and T cells (CD90/Thy1.2⁺), confirming flow cytometry analyses (Fig. 3*b* and data not shown). No B220 staining was observed in lung sections. Lung sections from mice that received unlabelled decoys were used as controls. No staining was observed in these sections (Fig. 3).

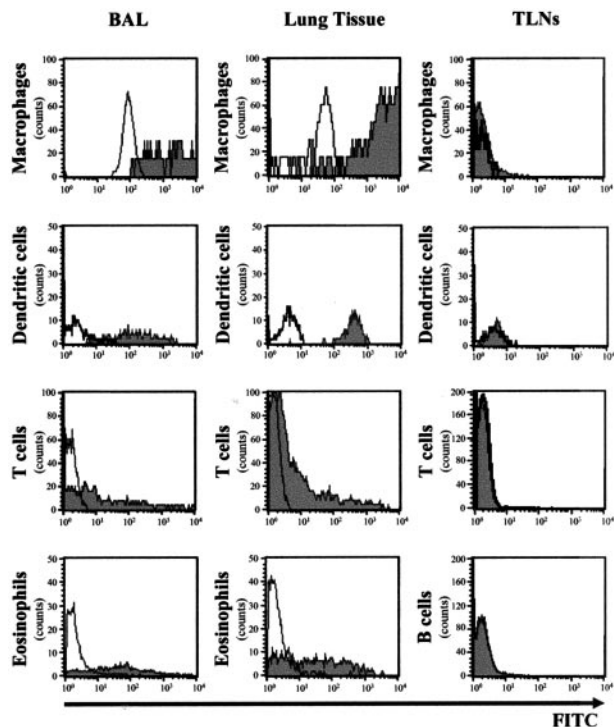


FIGURE 2. NF- κ B decoy ODNs target airway immune cells. Mice sensitized (days 0 and 14) and challenged (days 28–30) with OVA received NF- κ B decoy ODNs intratracheally on day 28 and day 30 (6 h before OVA challenge). The last instillation of NF- κ B decoys (day 30) was performed with FITC-labeled ODNs. At 24 h later (day 31), dendritic cells (I-A^{bhi}CD11c^{med-hi}), macrophages (F4/80⁺), eosinophils (large and granular CCR3⁺ cells), T cells (CD3 ϵ ⁺), and B cells (CD19⁺) from BALF, lung tissue, and TLNs were assessed for FITC positivity using flow cytometry (gray histograms). Cells from mice that received unlabeled decoys were used as controls (open histograms). These results represent at least three comparable experiments.

In vivo distribution of intratracheally administered scrambled ODNs, as assessed by flow cytometry and immunohistochemistry, was similar to that of NF- κ B decoys (data not shown). These results indicate that decoy ODNs inhibit NF- κ B activation in the airways of OVA/OVA mice by targeting local immune cells but not constitutive lung cells and draining lymph node cells.

NF- κ B decoy ODNs do not induce apoptosis of airway immune cells

Total NF- κ B inhibition triggers immune cell apoptosis (20–24). We therefore examined using Annexin V^{FITC} staining and flow cytometry analyses whether decoy-mediated NF- κ B inhibition was sufficient to induce apoptosis of lung immune cells in OVA/OVA mice. Twenty-four hours after the last OVA challenge (day 31), the rates of dendritic cell, macrophage, eosinophil, and T cell apoptosis were low in BALF and lung tissue of decoy-treated OVA/OVA mice (Table I). Moreover, these rates were similar to those recorded in untreated OVA/OVA animals (Table I). Thus, our results show that treatment of OVA/OVA mice with NF- κ B decoys does not induce apoptosis of airway immune cells.

NF- κ B decoys attenuate allergic lung inflammation, airway hyperresponsiveness and mucus production, but not specific Ig production, in OVA-challenged mice

We next analyzed whether intratracheal delivery of NF- κ B decoys would affect allergic airway inflammation, AHR, and mucus and OVA-specific Ig production in OVA/OVA mice.

Sham/OVA mice did not develop pulmonary inflammation upon OVA challenge, as revealed by histological examination (Fig. 4a, top left panel). Conversely, widespread peribronchiolar and perivascular inflammatory infiltrates were observed in OVA-sensitized mice after Ag provocation (Fig. 4a, top right panel). Lung-infiltrating cells were mostly eosinophils and lymphocytes. Treatment of OVA/OVA mice with NF- κ B decoys resulted in significant reduction of peribronchial and perivascular eosinophilic and lymphocytic inflammation (Fig. 4a, bottom right panel), whereas administration of scrambled ODNs had no detectable effects (Fig. 4a, bottom left panel).

Concomitant with histological assessment, airway inflammation was evaluated in the BALF (Fig. 4b). The total cell count in the BALF recovered from OVA/OVA mice was ~19-fold higher than that in the BALF derived from sham/OVA mice. This difference was mainly due to a drastic increase in eosinophil percentage in the BALF from OVA/OVA mice. Also, lymphocyte and macrophage numbers were significantly higher in these mice compared with sham/OVA animals. As shown in Fig. 4b, eosinophilia was strongly decreased in mice treated with NF- κ B decoys compared with untreated OVA/OVA mice. In addition to a blunted eosinophil recruitment, lymphocyte and macrophage recruitment was also significantly attenuated in the airways of decoy-treated mice. By contrast, treatment with scrambled ODNs had no significant effect on eosinophil, lymphocyte, and macrophage influx.

Bronchial epithelial cells in OVA/OVA mice showed extensive PAS positive staining indicating the presence of mucin collections (Fig. 5, top right panel). By contrast, sham/OVA mice had minimal to absent mucus staining (Fig. 5, bottom left panel). Treatment of OVA/OVA mice with NF- κ B decoys resulted in abrogation of mucus production (Fig. 5, bottom right panel), whereas administration of scrambled ODNs had no effects (Fig. 5, bottom left panel).

We further measured AHR to determine whether instillation of NF- κ B decoys influences allergen-induced lung dysfunction. For that, responsiveness to MCh was assessed by using whole-body plethysmography and increases in Penh as an index of airway obstruction. OVA-sensitized mice showed a drastic increase in AHR compared with sham/OVA animals after challenge (Fig. 6). Furthermore, it was found that administration of decoy but not scrambled ODNs leads to a significant reduction of AHR in OVA/OVA mice.

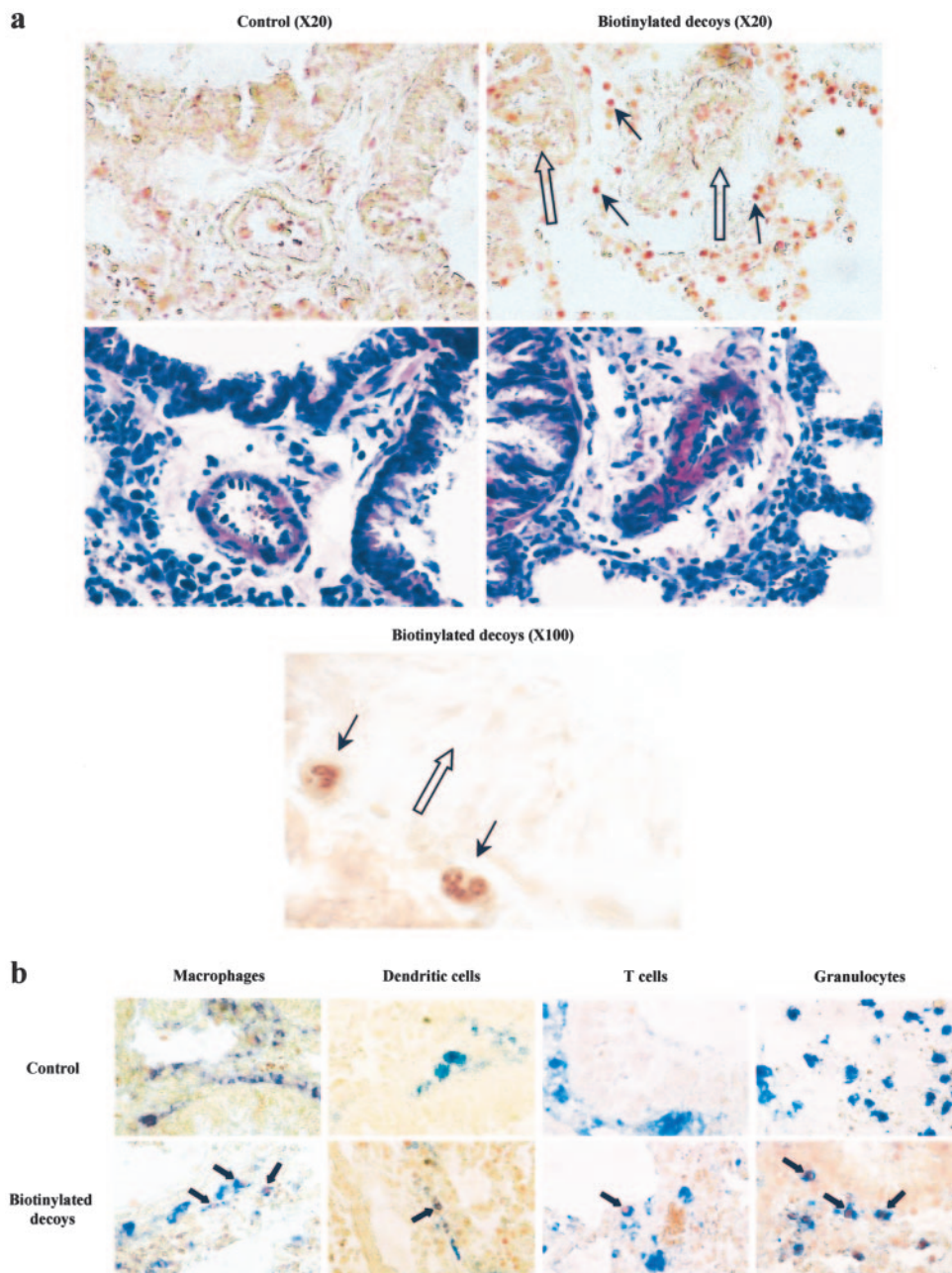
Serum levels of total IgE and OVA-specific IgE and IgG1 were elevated in OVA/OVA mice compared with sham/OVA animals (Fig. 7). OVA-specific IgG2a were undetectable in the samples. Of note, neither NF- κ B decoys nor scrambled ODNs modified serum Ig levels in OVA/OVA mice.

These findings show that treatment with NF- κ B decoy ODNs strongly attenuates allergic airway inflammation, AHR and mucus production, but not specific Ig production, in OVA/OVA mice.

Treatment with NF- κ B decoys reduces IL-5, IL-13 and eotaxin, but not IL-4, production in the airways of OVA-challenged mice

We next measured Th1 (IFN- γ and IL-2) and Th2 (IL-4, IL-5, and IL-13) cytokine concentrations in the BALF recovered from the animals. BALF levels of eotaxin, a potent chemoattractant for eosinophils and Th2 cells that depends on NF- κ B for its expression (25–31), were also determined. As expected in this model, BALF levels of eotaxin and Th2 but not Th1 cytokines were markedly increased in OVA/OVA mice compared with sham/OVA controls (Fig. 8). IL-5, IL-13, and eotaxin concentrations in the BALF of decoy-treated OVA/OVA mice were all significantly lower than those recorded in untreated OVA/OVA animals. Conversely, treatment with NF- κ B decoys did not affect BALF levels of IL-4. No

FIGURE 3. NF- κ B decoy ODNs target the nucleus of airway immune cells. Mice sensitized (days 0 and 14) and challenged (days 28–30) with OVA received NF- κ B decoy ODNs intratracheally on day 28 and day 30 (6 h before OVA challenge). The last ODN administration was performed on day 30 with biotinylated NF- κ B decoys, which were localized 24 h later using immunohistochemistry. *a*, Biotinylated NF- κ B decoys targeted peribronchial and perivascular immune cells (solid arrows, *top right panel*) at magnification $\times 20$ and were mainly located in the nucleus of these cells (solid arrows, *bottom panel*) at magnification $\times 100$. Constitutive lung cells did not incorporate biotinylated ODNs (open arrows, *top right panel* and *bottom panel*). Lung sections from mice that received unlabelled decoys were used as controls (*top left panel*). H&E staining of adjacent sections has been performed (*middle panels*) at magnification $\times 20$. *b*, Double staining showed that the majority of the signal was located in the nucleus of peribronchial and perivascular dendritic cells (I-E $^{k+}$), macrophages (F4/80 $^{+}$), granulocytes (Gr-1 $^{+}$), and T cells (CD90/Thy1.2 $^{+}$) (*bottom panels*), magnification $\times 20$. Lung sections from mice that received unlabelled decoys were used as controls (*top panels*) at magnification $\times 20$. These results represent at least three comparable experiments.



changes were seen after administration of scrambled ODNs (Fig. 8). These data show that intratracheal delivery of NF- κ B decoys significantly reduces IL-5, IL-13, and eotaxin, but not IL-4, production in the lungs of OVA/OVA mice.

Treatment of OVA/OVA mice with NF- κ B decoys does not affect the ability of TLN cells to produce Th2 cytokines in response to the allergen

We finally measured Th2 cytokine concentrations in culture supernatants from OVA-restimulated TLN cells. TLN cells from sham/OVA mice produced only low amounts of IL-4, IL-5 and IL-13 in the presence of OVA (Fig. 9). In contrast, stimulation of TLN cells from OVA/OVA animals with Ag markedly promoted the production of Th2 cytokines. The levels of IL-4, IL-5, and IL-13 produced in OVA-stimulated TLN cultures from decoy- and scrambled-treated OVA/OVA mice were similar to those observed in untreated OVA/OVA animals. Th2 cytokines were undetectable in supernatants from TLN cells cultured in the absence of OVA

(data not shown). Altogether, these results demonstrate that intratracheal administration of NF- κ B decoys to OVA/OVA mice does not alter the capacity of their TLN cells to secrete Th2 cytokines following Ag stimulation.

Discussion

Knockout mice studies have revealed that NF- κ B plays a critical role in Th2 cell differentiation and is therefore required for induction of allergic airway inflammation (9–11). However, it remains unclear whether NF- κ B also plays a role in the effector phase of airway allergy and whether inhibiting NF- κ B could have therapeutic value in the treatment of established asthma. To answer these questions, we have assessed in OVA-sensitized wild-type mice the effects of specifically antagonizing NF- κ B activity in the airways during OVA provocation. We report that intratracheal administration of decoy ODNs, but not scrambled controls, abrogated NF- κ B activation in the whole lung and in BALF cells following OVA challenge. NF- κ B inhibition was associated with

Table I. Percentage of apoptotic (annexin V⁺) immune cells in the BALF and lung tissue of untreated and NF- κ B decoy-treated OVA/OVA mice

	BALF		Lung Tissue	
	OVA/OVA	OVA/OVA + Decoys	OVA/OVA	OVA/OVA + Decoys
Macrophages (%)	6 $\pm$ 2	2.7 $\pm$ 2.4	2 $\pm$ 0.7	1 $\pm$ 0.4
Dendritic cells (%)	10 $\pm$ 5.3	7.7 $\pm$ 1.6	6.7 $\pm$ 2.2	9.3 $\pm$ 4.2
T cells (%)	0.8 $\pm$ 0.2	1.3 $\pm$ 0.4	4.3 $\pm$ 1.4	4.7 $\pm$ 2.2
Eosinophils (%)	5.3 $\pm$ 2.2	2.7 $\pm$ 2.1	4.3 $\pm$ 3.1	4 $\pm$ 2.7

strong attenuation of allergic lung inflammation, AHR and mucus production. This study demonstrates for the first time that local NF- κ B activation is critically involved in the effector phase of allergic airway disease and that pharmacologic inhibition of

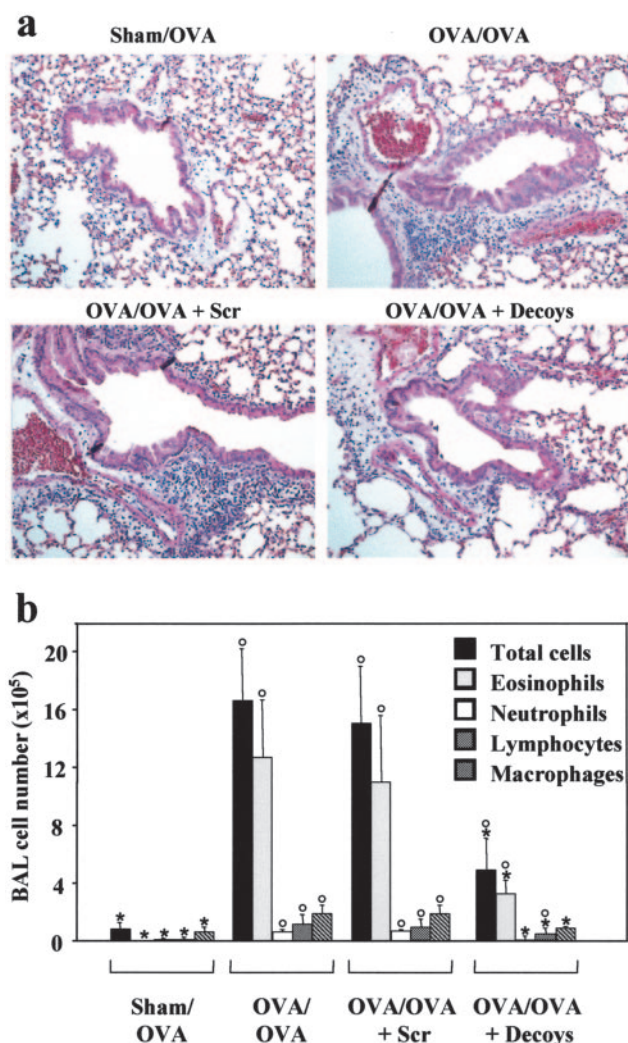


FIGURE 4. Treatment with NF- κ B decoys markedly attenuates allergic airway inflammation in experimental asthma. *a*, Lung sections of sham/OVA mice, untreated OVA/OVA mice, and OVA/OVA mice treated with either NF- κ B decoys or scrambled (Scr) ODNs were stained with H&E. *b*, The BALF recovered from the animals was subjected to total and differential cell counts. Data are presented as means $\pm$ SDs ($n = 6$ in each mice group). *, Significantly different from the results obtained in OVA/OVA mice. $\circ$, Significantly different from the results obtained in sham/OVA mice. These results represent six comparable experiments.

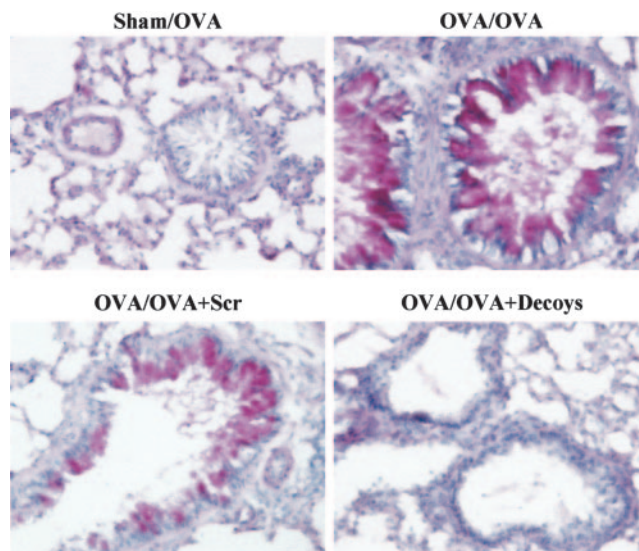


FIGURE 5. Treatment with NF- κ B decoys abrogates mucus production in OVA/OVA mice. Lung sections of sham/OVA mice, untreated OVA/OVA mice, and OVA/OVA mice treated with either NF- κ B decoys or scrambled (Scr) ODNs were stained with PAS (magenta) to assess intra-cytoplasmic and luminal mucin production. Comparable results were obtained in six experiments.

NF- κ B in the lungs has therapeutic potential in the control of pulmonary allergy.

The decoy approach has been used successfully in several animal models of human diseases and is now gaining increasing interest (see review Ref. 16). Usually, decoy ODNs are encapsulated in a fusogenic liposome or mixed with cationic lipids before being administered (16, 32). This procedure improves cellular uptake and nuclear localization, a prerequisite for optimal decoy function (16). However, it has been demonstrated that systemic or local injection of “naked” NF- κ B decoys may be sufficient to achieve NF- κ B inhibition *in vivo* and therefore prevention of experimentally induced inflammatory conditions (33, 34). In the present report, we show that intratracheal administration of “naked” NF- κ B decoys to OVA-sensitized mice results in efficient nuclear transfection of airway immune cells, thus precluding NF- κ B activation upon OVA stimulation. Bronchial epithelium did not incorporate NF- κ B decoys, an observation consistent with a previous report that intratracheal administration of NF- κ B decoys does not result in efficient transfection of airway epithelial cells and thus fail in preventing bleomycin-induced lung inflammation (35). Interestingly, nuclei of hepatocytes can be transfected very efficiently with decoy ODNs (36, 37), emphasizing that uptake of decoy ODNs is cell type-specific and that, as for plasmid-mediated gene transfer, airway epithelium appears to be a well-defended site for ODN-based therapy. The mechanisms responsible for cell type-specific differences in decoy ODN uptake are presently unknown and would require additional studies. Four lines of evidence support the specificity of our ODN-based approach. First, scrambled ODNs did not alter NF- κ B activity. Second, NF- κ B decoys did not affect Oct-1-binding activity. Third, NF- κ B decoys did not induce apoptosis of targeted cells, as determined by staining with annexin V-FITC and flow cytometry analyses. Fourth, the NF- κ B decoy ODNs used in the present study did not contain any of the currently recognized immunomodulatory sequences, including the antisense ODN for the HIV *rev* gene, the conventional CpG motif (TCG)_n or (CG)_n repeats, or palindrome sequences (38–42). We conclude that intratracheal delivery of “naked” decoy ODNs offers

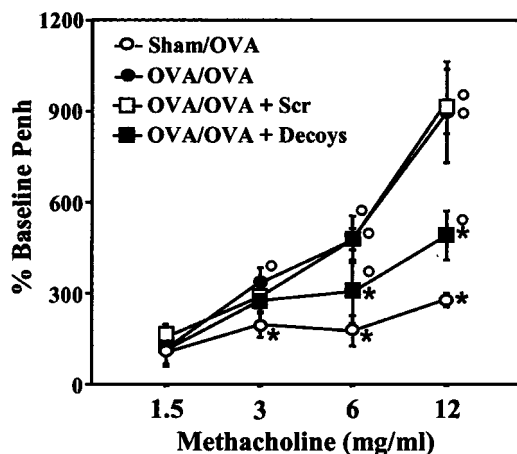


FIGURE 6. Intratracheal administration of NF- κ B decoys strongly reduces AHR in OVA/OVA mice. AHR was assessed in sham/OVA mice, untreated OVA/OVA mice, and OVA/OVA mice treated with either NF- κ B decoys or scrambled (Scr) ODNs, by analyzing Penh responses in a body plethysmograph. PBS or increasing concentrations of MCh (1.5–12 mg/ml) were nebulized for 3 min, and Penh measurements were taken and averaged for 3 min after each nebulization. Data are expressed as the fold increase above PBS challenge values. Data are presented as means $\pm$ SDs ($n = 6$ in each mice group). *, Significantly different from the results obtained in OVA/OVA mice. $\circ$, Significantly different from the results obtained in sham/OVA mice. Comparable results were obtained in four experiments.

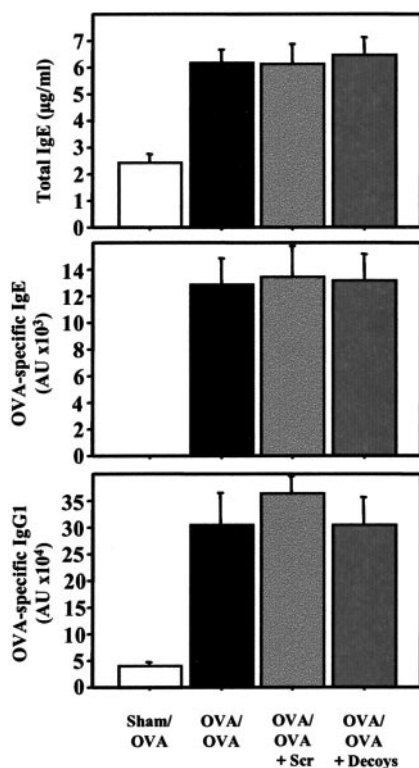


FIGURE 7. NF- κ B decoys do not affect specific Ig production in OVA-challenged mice. Serum samples were taken from sham/OVA mice, untreated OVA/OVA mice, and OVA/OVA mice treated with either NF- κ B decoys or scrambled (Scr) ODNs. Levels of total IgE and OVA-specific IgE and IgG1 were measured in these samples by ELISA. OVA-specific IgE and IgG1 levels are expressed using arbitrary units (AU). Data are presented as means $\pm$ SDs ($n = 6$ in each mice group). All the values obtained in OVA/OVA mice were significantly different from those obtained in sham/OVA mice. These results are similar to at least four comparable experiments.

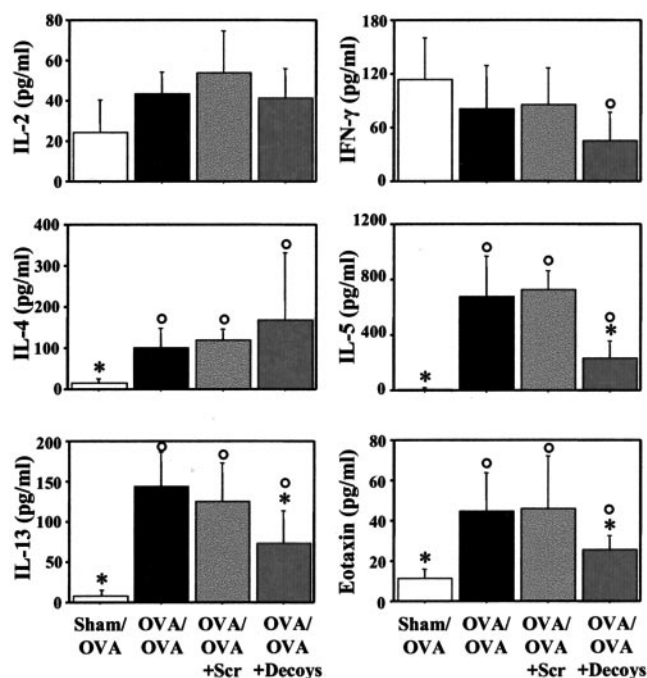


FIGURE 8. NF- κ B decoys inhibit IL-5, IL-13 and eotaxin, but not IL-4, production in the airways of OVA-challenged mice. Eotaxin, and Th1 (IFN- γ and IL-2) and Th2 (IL-4, IL-5, and IL-13) cytokine concentrations were measured in the BALF of sham/OVA mice, OVA/OVA mice, and OVA/OVA mice treated with either NF- κ B decoys or scrambled (Scr) ODNs. Cytokine levels were determined by specific ELISA and are presented as means $\pm$ SDs ($n = 6$ in each mice group). *, Significantly different from the results obtained in OVA/OVA mice. $\circ$, Significantly different from the results obtained in sham/OVA mice. These results are representative of at least six similar experiments.

an interesting means to specifically inhibit transcription factors in lung immune cells, both for basic research into the role of these factors and for development of new treatments for immunological airway diseases.

Recently, immunohistochemistry studies have demonstrated increased NF- κ B activity in bronchial epithelium and airway immune cells of OVA/OVA mice as compared with sham/OVA animals (43, 44). In the present study, although NF- κ B decoys only targeted airway immune cells (i.e., granulocytes, APCs and lymphocytes), treatment of OVA/OVA mice with decoys led to abrogation of NF- κ B activation in the whole lung. This observation is consistent with our previous report that the presence in the bronchi of inflammatory cells displaying NF- κ B activity, and thus secreting high levels of the proinflammatory cytokines TNF- α and IL-1 β , is required for the maintenance of NF- κ B activity in the epithelium of asthmatic airways (45).

Several lines of evidence suggest that NF- κ B decoys did not affect the secondary immune response that takes place in TLNs upon OVA challenge. First, lymph node cells did not incorporate NF- κ B decoys. Second, in vitro restimulation of lymph node cells from decoy-treated mice resulted in IL-5, IL-4, and IL-13 up-regulation similar to that observed in untreated mice. Third, IgE and IgG1 production was unaffected in decoy-treated animals. These findings support the hypothesis that intratracheally administered NF- κ B decoys caused severe blunting of allergic lung inflammation and AHR through their ability to target immune cells that invade the airways rather than by interfering with the immune response that occurs in TLNs.

As a result of NF- κ B inhibition in lung immune cells, IL-13, IL-5, and eotaxin expression was markedly reduced in the airways

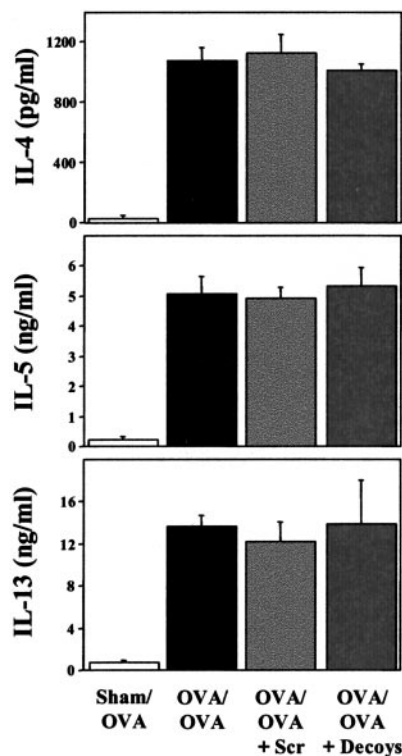


FIGURE 9. Treatment with NF- κ B decoys does not affect the capacity of TLN cells to produce Th2 cytokines when stimulated with OVA. TLN cells were isolated from sham/OVA mice, OVA/OVA mice, and OVA/OVA mice treated with either NF- κ B decoys or scrambled (Scr) ODNs. These cells were then cultured in the presence of 100 μ g/ml OVA for 96 h, after which time culture supernatants were harvested. IL-4, IL-5, and IL-13 levels in supernatants were determined by ELISA and are presented as means $\pm$ SDs ($n = 6$ in each mice group). All the values obtained in OVA/OVA mice were significantly different from those obtained in sham/OVA mice. Similar results were obtained in at least six experiments.

of decoy-treated mice as compared with untreated OVA/OVA animals. This observation is consistent with previous findings that immune cells are important or exclusive producers of these cytokines (see review Refs. 5, 46, 47) and that the 5'-flanking region in the genes encoding IL-13 and eotaxin contain putative NF- κ B binding sites (30, 48). IL-13 mediates eosinophil egression, AHR and mucus overproduction (2–4, 49–52). IL-5 promotes eosinophil differentiation, proliferation, activation and survival, and thus plays an essential role in initiation and persistence of airway eosinophilia (53–59). Lastly, eotaxin has been shown to be a specific chemoattractant for eosinophils and Th2 cells (25–28). Thus, given the respective roles of IL-13, IL-5, and eotaxin in allergic responses, the combined deficiency of all these cytokines in the airways of NF- κ B decoy-treated OVA/OVA mice could explain the significant attenuation of AHR, allergic inflammation, and mucus production in these animals. Moreover, it is reasonable to speculate that reduced immune cell recruitment in the airways may, in turn, account for attenuation of cytokine production. Our data provide the first in vivo demonstration that NF- κ B is essentially required for local IL-5, IL-13, and eotaxin expression, and thus for occurrence of AHR, allergic inflammation and mucus production, during the effector phase of pulmonary allergy.

Surprisingly, local IL-4 production did not decrease in OVA/OVA mice following decoy treatment, despite reduced accumulation of IL-4-producing cells, namely Th2 cells and eosinophils (46, 47), in the airways of these animals. A possibility is that NF- κ B directly or indirectly represses IL-4 gene expression, and that

NF- κ B inhibition leads to exaggerated IL-4 expression in Th2 cells and eosinophils that invade the lungs of decoy-treated mice. This hypothesis is consistent with a previous report that the p50 homodimer, the activation of which was inhibited in our study, may function as a direct repressor of gene expression (60). Additional studies are needed to clarify this issue.

Although prototypical Th2 cytokines are often coordinately expressed, selective production of either IL-4 or IL-5 has been reported in several pathological situations (see review Ref. 61). An example is intrinsic or nonatopic asthma, with eosinophilic bronchitis but without elevated IgE production, where there is evidence for excessive expression of IL-5 but not IL-4 (62). It has been hypothesized that differential transcription factor activation or inhibition could cause differential Th2 cytokine expression (3). Consistent with this assumption, we show that intact NF- κ B activity in immune cells present at the site of allergic inflammation is required to ensure coincidence of effector Th2 responses.

Corticosteroids are currently the mainstay of therapy in asthma (see reviews Refs. 63, 64). Steroids suppress the expression of multiple genes encoding cytokines, chemokines, adhesion molecules and inflammatory enzymes, and this is why they are so effective in controlling allergic inflammation (63, 64). Consistent with this assertion is the fact that therapeutic strategies based on blocking individual proinflammatory mediators, for example antagonism of IL-5, have proven ineffective (65). Most of the anti-inflammatory actions of corticosteroids can be accounted for by inhibiting transcription factors, mainly NF- κ B and activator protein 1 that regulate inflammatory gene expression (transrepression) (see reviews Refs. 64, 66). In contrast, systemic side effects of corticosteroids are mediated through activation of gene expression (transactivation), prompting a search for steroids that would be able to induce transrepression only (67–70). When designing the present study, we postulated that direct inhibition of NF- κ B could be an alternative way to selectively reproduce the key anti-inflammatory actions of corticosteroids. We report that local NF- κ B inhibition is associated with strong attenuation of cytokine expression, mucus production, eosinophilic and lymphocytic inflammation, and AHR in experimental asthma, reinforcing our hypothesis.

Acknowledgments

We thank Drs. Fabienne Andris, Pierre Châtelain, Bruno Fuks, Oberdan Leo, Roy Massingham, Muriel Moser, and André-Bernard Tonnel for advice, Virginie Garze, Fabrice Jaspas, Martine Leblond, Philippe Marquillies, Dorothée Mélotte, Ilham Sbaï, and Marjorie Vermeersch for excellent technical and secretarial assistance. This article is dedicated to the memory of our colleague Laurent Fraga.

References

- Viegi, G., I. Annesi, and G. Matteelli. 2003. Epidemiology of asthma. *Eur. Respir. Mon.* 23:1.
- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17:255.
- Ray, A., and L. Cohn. 1999. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *J. Clin. Invest.* 104:985.
- Busse, W. W., and R. F. Lemanske. 2001. Asthma. *N. Engl. J. Med.* 344:350.
- Larche, M., D. S. Robinson, and A. B. Kay. The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 111:450.
- Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16:225.
- Hart, L. A., V. L. Krishnan, I. M. Adcock, P. J. Barnes, and K. F. Chung. 1998. Activation and localization of transcription factor, nuclear factor- κ B, in asthma. *Am. J. Respir. Crit. Care Med.* 158:1585.
- Bureau, F., G. Bonizzi, N. Kirschvink, S. Delhalle, D. Desmecht, M. P. Merville, V. Bours, and P. Lekeux. 2000. Correlation between nuclear factor- κ B activity in

- bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am. J. Respir. Crit. Care Med.* 161:1314.
9. Yang, L., L. Cohn, D. H. Zhang, R. Homer, A. Ray, and P. Ray. 1998. Essential role of nuclear factor κ B in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med.* 188:1739.
 10. Das, J., C. H. Chen, L. Yang, L. Cohn, P. Ray, and A. Ray. 2001. A critical role for NF- κ B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat. Immunol.* 2:45.
 11. Donovan, C. E., D. A. Mark, H. Z. He, H. C. Liou, L. Kobzik, Y. Wang, G. T. De Sanctis, D. L. Perkins, and P. W. Finn. 1999. NF- κ B/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. *J. Immunol.* 163:6827.
 12. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587.
 13. Zhang, D. H., L. Yang, L. Cohn, L. Parkyn, R. Homer, P. Ray, and A. Ray. 1999. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity* 11:473.
 14. Bielinska, A., R. A. Shivdasani, L. Q. Zhang, and G. J. Nabel. 1990. Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. *Science* 250:997.
 15. Sullenger, B. A., H. F. Gallardo, G. E. Ungers, and E. Gilboa. 1990. Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. *Cell* 63:601.
 16. Mann, M. J., and V. J. Dzau. 2000. Therapeutic applications of transcription factor decoy oligonucleotides. *J. Clin. Invest.* 106:1071.
 17. Morishita, R., T. Sugimoto, M. Aoki, I. Kida, N. Tomita, A. Moriguchi, K. Maeda, Y. Sawa, Y. Kaneda, J. Higaki, and T. Ogihara. 1997. In vivo transfection of *cis* element "decoy" against nuclear factor- κ B binding site prevents myocardial infarction. *Nat. Med.* 3:894.
 18. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766.
 19. Pajak, B., T. De Smedt, V. Moulin, C. De Trez, R. Maldonado-Lopez, G. Vansanten, E. Briand, J. Urbain, O. Leo, and M. Moser. 2000. Immunohistochemistry, a new fixation and embedding method for light microscopy, which preserves antigen immunoreactivity and morphological structures: visualisation of dendritic cells in peripheral organs. *J. Clin. Pathol.* 53:518.
 20. Wu, M., H. Lee, R. E. Bellas, S. L. Schauer, M. Arsur, D. Katz, M. J. FitzGerald, T. L. Rothstein, D. H. Sherr, and G. E. Sonenshein. 1996. Inhibition of NF- κ B/Rel induces apoptosis of murine B cells. *EMBO J.* 15:4682.
 21. Kolenko, V., T. Bloom, P. Rayman, R. Bukowski, E. Hsi, and J. Finke. 1999. Inhibition of NF- κ B activity in human T lymphocytes induces caspase-dependent apoptosis without detectable activation of caspase-1 and -3. *J. Immunol.* 163:590.
 22. Ward, C., E. R. Chilvers, M. F. Lawson, J. G. Pryde, S. Fujihara, S. N. Farrow, C. Haslett, and A. G. Rossi. 1999. NF- κ B activation is a critical regulator of human granulocyte apoptosis in vitro. *J. Biol. Chem.* 274:4309.
 23. Pagliari, L. J., H. Perlman, H. Liu, and R. M. Pope. 2000. Macrophages require constitutive NF- κ B activation to maintain A1 expression and mitochondrial homeostasis. *Mol. Cell Biol.* 20:8855.
 24. Bureau, F., A. Vanderplasse, F. Jaspard, F. Minner, P. P. Pastoret, M. P. Merville, V. Bours, and P. Lekeux. 2002. Constitutive nuclear factor- κ B activity preserves homeostasis of quiescent mature lymphocytes and granulocytes by controlling the expression of distinct Bcl-2 family proteins. *Blood* 99:3683.
 25. Jose, P. J., I. M. Adcock, D. A. Griffiths-Johnson, N. Berkman, T. N. Wells, T. J. Williams, and C. A. Power. 1994. Eotaxin: cloning of an eosinophil chemoattractant cytokine and increased mRNA expression in allergen-challenged guinea-pig lungs. *Biochem. Biophys. Res. Commun.* 205:788.
 26. Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med.* 179:881.
 27. Rothenberg, M. E., A. D. Luster, and P. Leder. 1995. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc. Natl. Acad. Sci. USA* 92:8960.
 28. Ponath, P. D., S. Qin, D. J. Ringler, J. Clark-Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J. A. Gonzalo, W. Newman, et al. 1996. Cloning of the human eosinophil chemoattractant, eotaxin: expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J. Clin. Invest.* 97:604.
 29. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 277:2005.
 30. Hein, H., C. Schluter, R. Kulke, E. Christophers, J. M. Schroder, and J. Bartels. 1997. Genomic organization, sequence, and transcriptional regulation of the human eotaxin gene. *Biochem. Biophys. Res. Commun.* 237:537.
 31. Matsukura, S., C. Stellato, J. R. Plitt, C. Bickel, K. Miura, S. N. Georas, V. Casolaro, and R. P. Schleimer. 1999. Activation of eotaxin gene transcription by NF- κ B and STAT6 in human airway epithelial cells. *J. Immunol.* 163:6876.
 32. Morishita, R., G. H. Gibbons, M. Horiuchi, K. E. Ellison, M. Nakama, L. Zhang, Y. Kaneda, T. Ogihara, and V. J. Dzau. 1995. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo. *Proc. Natl. Acad. Sci. USA* 92:5855.
 33. Abeyama, K., W. Eng, J. V. Jester, A. A. Vink, D. Edelbaum, C. J. Cockerell, P. R. Bergstresser, and A. Takashima. 2000. A role for NF- κ B-dependent gene transactivation in sunburn. *J. Clin. Invest.* 105:1751.
 34. D'Acquisto, F., A. Ialenti, A. Ianaro, R. Di Vaio, and R. Carnuccio. 2000. Local administration of transcription factor decoy oligonucleotides to nuclear factor- κ B prevents carrageenin-induced inflammation in rat hind paw. *Gene Ther.* 7:1731.
 35. Griesenbach, U., R. L. Cassady, R. J. Cain, R. M. duBois, D. M. Geddes, and E. W. Alton. 2002. Cytoplasmic deposition of NF- κ B decoy oligonucleotides is insufficient to inhibit bleomycin-induced pulmonary inflammation. *Gene Ther.* 9:1109.
 36. Kren, B. T., P. Bandyopadhyay, and C. J. Steer. 1998. In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nat. Med.* 4:285.
 37. Kren, B. T., B. Parashar, P. Bandyopadhyay, N. R. Chowdhury, J. R. Chowdhury, and C. J. Steer. 1999. Correction of the UDP-glucuronosyltransferase gene defect in the Gunn rat model of Crigler-Najjar syndrome type I with a chimeric oligonucleotide. *Proc. Natl. Acad. Sci. USA* 96:10349.
 38. Branda, R. F., A. L. Moore, L. Mathews, J. J. McCormack, and G. Zon. 1993. Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1. *Biochem. Pharmacol.* 45:2037.
 39. Liang, H., Y. Nishioka, C. F. Reich, D. S. Pisetsky, and P. E. Lipsky. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* 98:1119.
 40. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546.
 41. Iho, S., T. Yamamoto, T. Takahashi, and S. Yamamoto. 1999. Oligodeoxynucleotides containing palindromic sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN- γ production in vitro. *J. Immunol.* 163:3642.
 42. Carson, D. A., and E. Raz. 1997. Oligonucleotide adjuvants for T helper 1 (Th1)-specific vaccination. *J. Exp. Med.* 186:1621.
 43. Poynter, M. E., C. G. Irvin, and Y. M. Janssen-Heininger. 2002. Rapid activation of nuclear factor- κ B in airway epithelium in a murine model of allergic airway inflammation. *Am. J. Pathol.* 160:1325.
 44. Pastva, A., K. Estell, T. R. Schoeb, T. P. Atkinson, and L. M. Schwiebert. 2004. Aerobic exercise attenuates airway inflammatory responses in a mouse model of atopic asthma. *J. Immunol.* 172:4520.
 45. Bureau, F., S. Delhalle, G. Bonizzi, L. Fievez, S. Dogne, N. Kirschvink, A. Vanderplasse, M. P. Merville, V. Bours, and P. Lekeux. 2000. Mechanisms of persistent NF- κ B activity in the bronchi of an animal model of asthma. *J. Immunol.* 165:5822.
 46. Williams, T. J., and P. J. Jose. 2000. Role of eotaxin and related CC chemokines in allergy and asthma. *Chem. Immunol.* 78:166.
 47. Borish, L. C., and J. W. 2003. Steinke. 2. Cytokines and chemokines. *J. Allergy Clin. Immunol.* 111:S460.
 48. McKenzie, A. N., X. Li, D. A. Largaespada, A. Sato, A. Kaneda, S. M. Zurawski, E. L. Doyle, A. Milatovich, U. Francke, and N. G. Copeland. 1993. Structural comparison and chromosomal localization of the human and mouse IL-13 genes. *J. Immunol.* 150:5436.
 49. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261.
 50. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258.
 51. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 103:779.
 52. Walter, D. M., J. J. McIntire, G. Berry, A. N. McKenzie, D. D. Donaldson, R. H. DeKruyff, and D. T. Umetsu. 2001. Critical role for IL-13 in the development of allergen-induced airway hyperactivity. *J. Immunol.* 167:4668.
 53. Lopez, A. F., C. J. Sanderson, J. R. Gamble, H. D. Campbell, I. G. Young, and M. A. Vadas. 1988. Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J. Exp. Med.* 167:219.
 54. Yamaguchi, Y., Y. Hayashi, Y. Sugama, Y. Miura, T. Kasahara, S. Kitamura, M. Torisu, S. Mita, A. Tominaga, and K. Takatsu. 1988. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival: IL-5 as an eosinophil chemotactic factor. *J. Exp. Med.* 167:1737.
 55. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med.* 167:43.
 56. Clutterbuck, E. J., E. M. Hirst, and C. J. Sanderson. 1989. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GM-CSF. *Blood* 73:1504.
 57. Nakajima, H., I. Iwamoto, S. Tomoe, R. Matsumura, H. Tomioka, K. Takatsu, and S. Yoshida. 1992. CD4⁺ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am. Rev. Respir. Dis.* 146:374.
 58. Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young, and K. I. Matthaei. 1996. IL-5-deficient mice have a developmental defect in CD5⁺ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* 4:15.
 59. Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183:195.

60. Kang, S. M., A. C. Tran, M. Grilli, and M. J. Lenardo. 1992. NF- κ B subunit regulation in nontransformed CD4⁺ T lymphocytes. *Science* 256:1452.
61. Sewell, W. A., and H. H. Mu. 1996. Dissociation of production of interleukin-4 and interleukin-5. *Immunol. Cell Biol.* 74:274.
62. Doi, S., V. Gemou-Engesaeth, A. B. Kay, and C. J. Corrigan. 1994. Polymerase chain reaction quantification of cytokine messenger RNA expression in peripheral blood mononuclear cells of patients with acute exacerbations of asthma: effect of glucocorticoid therapy. *Clin. Exp. Allergy* 24:854.
63. Barnes, P. J. 2000. New directions in allergic diseases: mechanism-based anti-inflammatory therapies. *J. Allergy Clin. Immunol.* 106:5.
64. Adcock, I. M. 2003. Glucocorticoids: new mechanisms and future agents. *Curr. Allergy Asthma Rep.* 3:249.
65. Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, et al. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356:2144.
66. Adcock, I. M. 2001. Glucocorticoid-regulated transcription factors. *Pulm. Pharmacol. Ther.* 14:211.
67. Vayssiere, B. M., S. Dupont, A. Choquart, F. Petit, T. Garcia, C. Marchandeu, H. Gronemeyer, and M. Resche-Rigon. 1997. Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. *Mol. Endocrinol.* 11:1245.
68. Belvisi, M. G., S. L. Wicks, C. H. Battram, S. E. Bottoms, J. E. Redford, P. Woodman, T. J. Brown, S. E. Webber, and M. L. Foster. 2001. Therapeutic benefit of a dissociated glucocorticoid and the relevance of in vitro separation of transrepression from transactivation activity. *J. Immunol.* 166:1975.
69. Reichardt, H. M., J. P. Tuckermann, M. Gottlicher, M. Vujic, F. Weih, P. Angel, P. Herrlich, and G. Schutz. 2001. Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *EMBO J.* 20:7168.
70. Schacke, H., W. D. Docke, and K. Asadullah. 2002. Mechanisms involved in the side effects of glucocorticoids. *Pharmacol. Ther.* 96:23.