Dexamethasone Does Not Inhibit Airway CXC Chemokine Expression and Neutrophilia in a Murine Model of Asthma - Mechanism of Steroid Resistance in Asthma

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ABSTRACT

Background: Although glucocorticoids (GCs) are effective in controlling asthma in the majority of patients, a subset of asthmatics fails to demonstrate a satisfactory response, even to systemic GC therapy. This population is referred to as being "steroid-resistant". The actual mechanism underlying steroid resistance in asthma remains to be elucidated. **Methods:** We have investigated how dexamethasone (DEX) regulates asthmatic phenotypes in a murine model of asthma, in which mice received i.p. immunization twice, followed by two bronchoprovocations with aerosolized OVA with a one-week interval, which we have recently described. **Results:** Pretreatment with DEX resulted in an inhibition of NF-κB activation in asthmatic lungs, and also inhibited broncho-alveolar lavage (BAL) levels of NF-κB-dependent cytokines such as TNF-α and CC chemokines [eotaxin and monocyte chemotactic protein (MCP)-1]. DEX was effective in suppressing airway hyperresponsiveness (AHR) at 10 h, Th2-dependent asthmatic phenotypes such as airway eosinophilia, BAL levels of Th2 cytokines (IL-5 and IL-13), and mucin production. However, DEX failed to suppress BAL levels of CXC chemokines [macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC)] and airway neutrophilia. **Conclusion:** Airway neutrophilia is among the phenomena observed in patients with severe GC-resistant asthma. This study will provide insight into the molecular basis for airway neutrophila seen in steroid-resistant asthma. Further studies are required to delineate the underlying mechanism of CXC chemokine expression in asthma. **(Immune Network 2007;7(1):18-25)**

Key Words: Asthma, steroid, NF-κB, CXC chemokine, neutrophilia

Introduction

Glucocorticoids (GCs) have been successfully used as anti-inflammatory and immunosuppressive agents in the treatment of chronic inflammatory diseases, including asthma (1). GCs have been reported to reduce the intensity of inflammation (2-5), diminish airway hyperresponsiveness (6,7), and have some impact on airway wall remodeling (2).

Although GCs are effective in controlling asthma in most patients, a subset of asthmatics fails to demonstrate a satisfactory response, even to systemic GC therapy (8,9). This population is referred to as being "steroid-resistant" (SR). These patients account for a large proportion of the high costs involved in the treatment of asthma (10). Impaired GC responsiveness has been studied most extensively in relation to asthma, but has also been reported in other inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and transplant rejection (11).

Several mechanisms have been proposed to account for a failure to respond to GCs. These include i) IL-2- and IL-4-induced resistance in which activated p38 MAP kinase phosphorylates GC receptors (GRs), which results in a reduction in corticosteroid binding affinity within the nucleus (12); ii) increased expression of GRβ, which may theoretically act as an inhibitor by competing with GRα for binding to GRE sites or by interacting with coactivator molecules (13); iii) a failure of GRs to inhibit the activation of inflammatory genes through transcription factors such as NF-κB and AP-1 (14,15); and

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iv) a defective histone acetylation, which may be associated with an impaired nuclear localization of GR (16). However, the actual mechanism by which GCs reduce airway inflammation in asthma remains poorly understood.

To gain further insight into potential mechanisms by which GCs act *in vivo*, we have investigated how dexamethasone (DEX) regulates asthmatic phenotypes in a previously described murine model of asthma (17).

Materials and Methods

Animals. Specific pathogen-free female BALB/c mice were obtained from Samtaco Inc. (Osan, Republic of Korea), housed in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. Mice were 7～8 weeks old at the start of each experiment. All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

Immunization and challenge. Mice were immunized i.p. with 20 μg of ovalbumin (OVA, grade V from Sigma) plus 2.25 mg aluminum hydroxide adjuvant on day 0 and OVA alone without alum on day 10 (17). The immunized mice were exposed to aerosolized OVA on days 17 and 24. Aerosolization of OVA was performed using a chamber that was adapted for mice. Animals were exposed to OVA (2.5% at first aerosolization and 5% at second aerosolization) using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan; output 0.8 ml/min) for 20 min in a Plexiglas exposure chamber (24.5×40.5×15.0 cm). Control animals received the same immunization and first airway challenge of OVA, but were exposed to aerosolized saline instead of OVA during the second airway challenge.

Bronchoalveolar lavage (BAL). BAL was performed at the time indicated after the second airway challenge as described previously (17).

Determination of airway hyperresponsiveness (AHR). AHR was assessed as a change in airway function after challenge with aerosolized methacholine via the airway, as described elsewhere (18,19). Anesthetization was achieved with 80 mg/kg of pentobarbital sodium injected intraperitoneally (i.p.). The trachea was then exposed through midcervical incision and tracheostomized, and an 18-gauge metal needle was then inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada), and each mouse was quasi-sinusoidally ventilated with a nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume similar to that occurring during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to a water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with increasing concentrations of methacholine (2.5～50 mg/ml in saline) in an aerosol form. The data needed to calculate R_L was collected continuously following each methacholine challenge. Maximum R_L values were selected to express changes in airway function, which was represented as a percent change from baseline after saline aerosol treatment.

Gel shift assay. Nuclear extracts were prepared from the lungs as described previously (20,21). To inhibit endogenous protease activity, 1 mM phenylmethylsulfonyl fluoride was added. As a probe for the gel retardation assay, an oligonucleotide containing the Ig κ-chain binding site (κB, 5'-CCG GTT AAC AGA GGG GGC TTT CCG AG-3') was synthesized. The two complementary strands were annealed and labeled with (α-^{32}P) dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris-HCl (pH 7.6), 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly (dIdC), and 1 mM DTT) were incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixture was analyzed by electrophoresis on a 5% polyacrylamide gel in 0.5X tris-borate/EDTA buffer. Specific binding was controlled by competition with a 50-fold excess of cold κB or cAMP response element (CRE) oligonucleotide. The signal intensities of specific bands were analyzed quantitatively using the Fluor-STM Imager (Bio-Rad, Muncher, Germany) and plotted as relative intensities.

Mucin analysis. Sections of fixed, embedded lung tissues were cut to 4 μm, placed on glass slides, and deparaffinized. Tissue samples were then stained with

periodic acid-Schiff (PAS).

Cytokine assays. IL-5, IL-13, monocyte chemotactic protein (MCP)-1, eotaxin, macrophage inflammatory protein (MIP)-2, keratinocyte-derived chemokine (KC), and TNF-α protein levels in BAL were determined by ELISA. The lower limits of detection for the cytokines were as follows: IL-5 (>5 pg/ml; R&D Systems), IL-13 (>1.5 pg/ml; R&D Systems), eotaxin (>3 pg/ml; R&D Systems), MCP-1 (>2 pg/ml; R&D Systems), MIP-2 (>1.5 pg/ml; R&D Systems), KC (>2.0 pg/ml; R&D Systems), TNF-α (>5.1 pg/ml; R&D Systems).

Statistical analysis. Data were expressed as mean±SD. Statistical comparison was performed using one-way ANOVA followed by the Fisher test. Significant differences between the groups were determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was accepted as an indication of statistical significance.

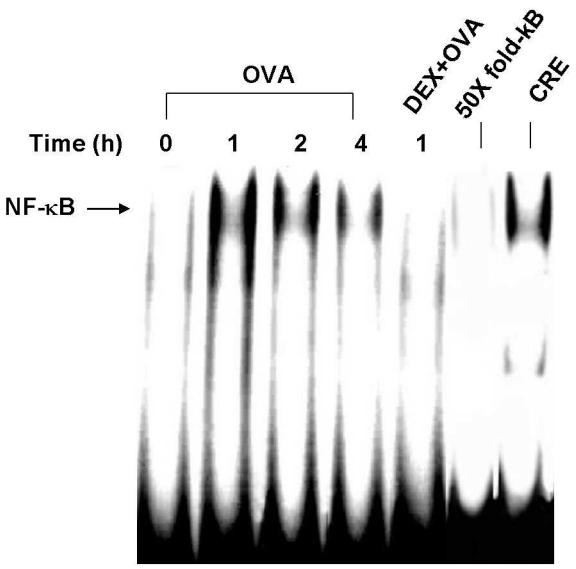

Figure 1. DEX inhibition of NF-κB activation in the lungs. Mice were sensitized and airway-challenged with OVA as described in Materials and Methods. Nuclear extracts of lung tissue obtained at the time indicated after second airway challenge were subjected to a gel shift assay for NF-κB activation. A 50-fold excess of κB or irrelevant CRE oligonucleotide was added for competition with the probe. Similar data were obtained from three independent experiments.

Results

DEX inhibits NF-κB activation in asthma. Mice were sensitized and immunized with OVA. The second airway challenge induced the activation of NF-κB in the lungs at 1 h (Fig. 1). Complete blocking of NF-κB mobilization by the addition of a cold competitor, but not by the addition of of an irrelevant motif, CRE, indicated the specificity of NF-κB binding. Pretreatment with DEX resulted in a significant inhibition of NF-κB activation (Fig. 1).

Effect of DEX on NF-κB-dependent asthmatic phenotypes. We determined the effect of DEX on NF-κB-dependent phenotypes such as i) BAL fluid levels of TNF-α and TNF-α-induced late AHR (24), ii) BAL fluid levels of CC chemokines (eotaxin and MCP-1) and CXC chemokines (KC and MIP-2), and iii) recruitment of neutrophils and macrophages into the airways. Induction of asthma resulted in increases in the BAL fluid level of TNF-α (Fig. 2A), TNF-α-mediated late AHR at 10 h (Fig. 2B), BAL fluid levels of CXC chemokines (Fig. 3A, B) and CC chemokines (Fig. 3C, D), and recruitment of neutrophils at 12 h (Fig. 4). DEX significantly suppressed the BAL level of TNF-α (Fig. 2A), as well as late AHR at 10 h

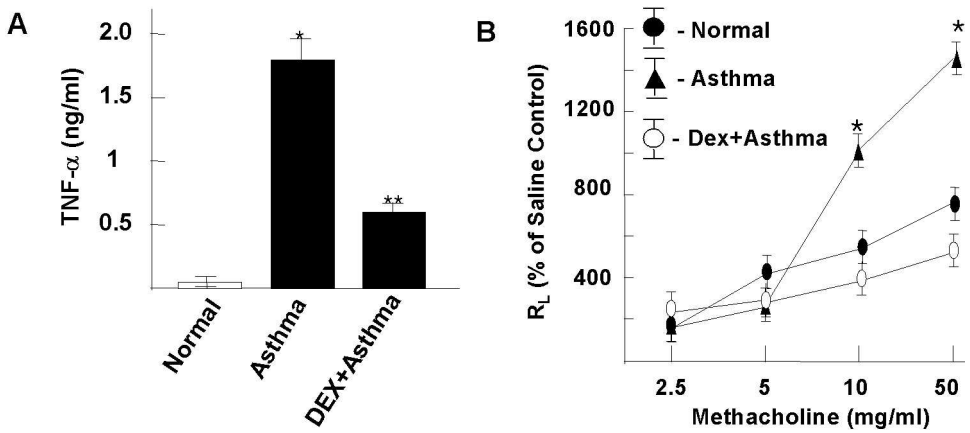

Figure 2. DEX inhibits the BAL level of TNF-α and late AHR. DEX (3.5 mg/kg) was given i.p. 1 day and 1 h prior to the second airway challenge. (A) BAL fluids were collected 1 h after the second airway challenge for measurement of TNF-α. (B) AHR was assessed 10 h after the second airway challenge. Results are expressed as mean±SD of three separate experiments (n=3~5 per group). *$p < 0.05$ vs. asthma group.

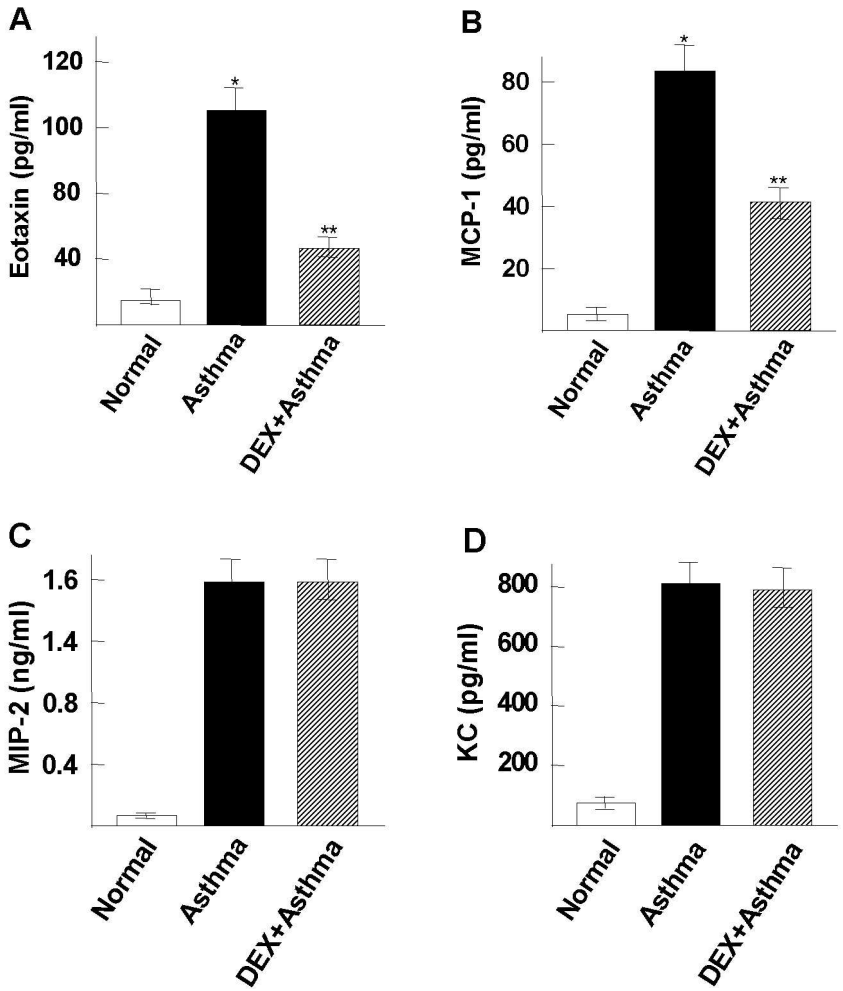

Figure 3. Effects of DEX on BAL chemokine levels. DEX (3.5 mg/kg) was given i.p. 1 day and 1 h prior to the second airway challenge. BAL fluids were collected 1 h after the second airway challenge for measurement of KC (A) and MIP-1 (B), and 12 h after the second airway challenge for measurement of eotaxin (C) and MCP-1 (D). Results are expressed as mean±SD of three separate experiments (n=3～5 per group). *$p<0.01$ vs. normal, **$p<0.01$ vs. asthma group.

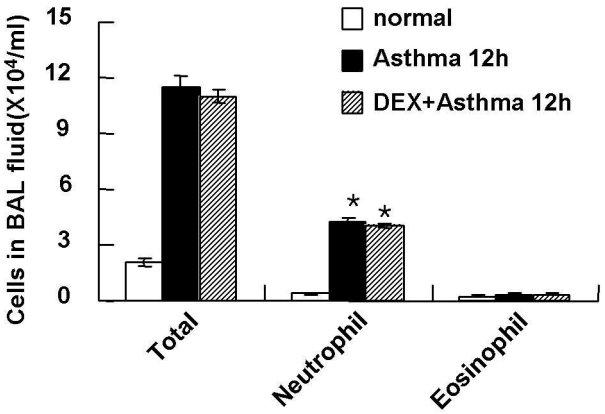

Figure 4. DEX fails to inhibit airway neutrophilia. DEX (3.5 mg/kg) was given i.p. 1 day and 1 h prior to the second airway challenge. BAL fluid numbers of neutrophils were assessed 12 h after the second airway challenge. Results are expressed as mean±SD of three separate experiments (n=3～5 per group). *$p<0.05$ vs. asthma group.

(Fig. 2B). DEX was also effective in inhibiting CC chemokines (Fig. 3A, B), but failed to suppress BAL levels of CXC chemokines (Fig. 3C, D) and airway neutrophilia (Fig. 4).

DEX inhibits Th2 cell-mediated asthmatic phenotypes. The effect of DEX on Th2 cell-mediated asthmatic phenotypes such as airway eosinophilia, BAL levels of Th2 cytokines (IL-5 and IL-13), and airway mucus production were examined. OVA-immunized and -challenged animals showed significant increases in the numbers of BAL eosinophils at 48 h after the second OVA challenge compared with saline-challenged controls (Fig. 5A). Pretreatment with DEX resulted in significant suppression of the recruitment of eosinophils by more than 80～90%. The levels of IL-5 and IL-13 in BAL fluids were significantly increased by airway challenge with OVA when compared with the saline-challenged control group (Fig. 5B). Pre-

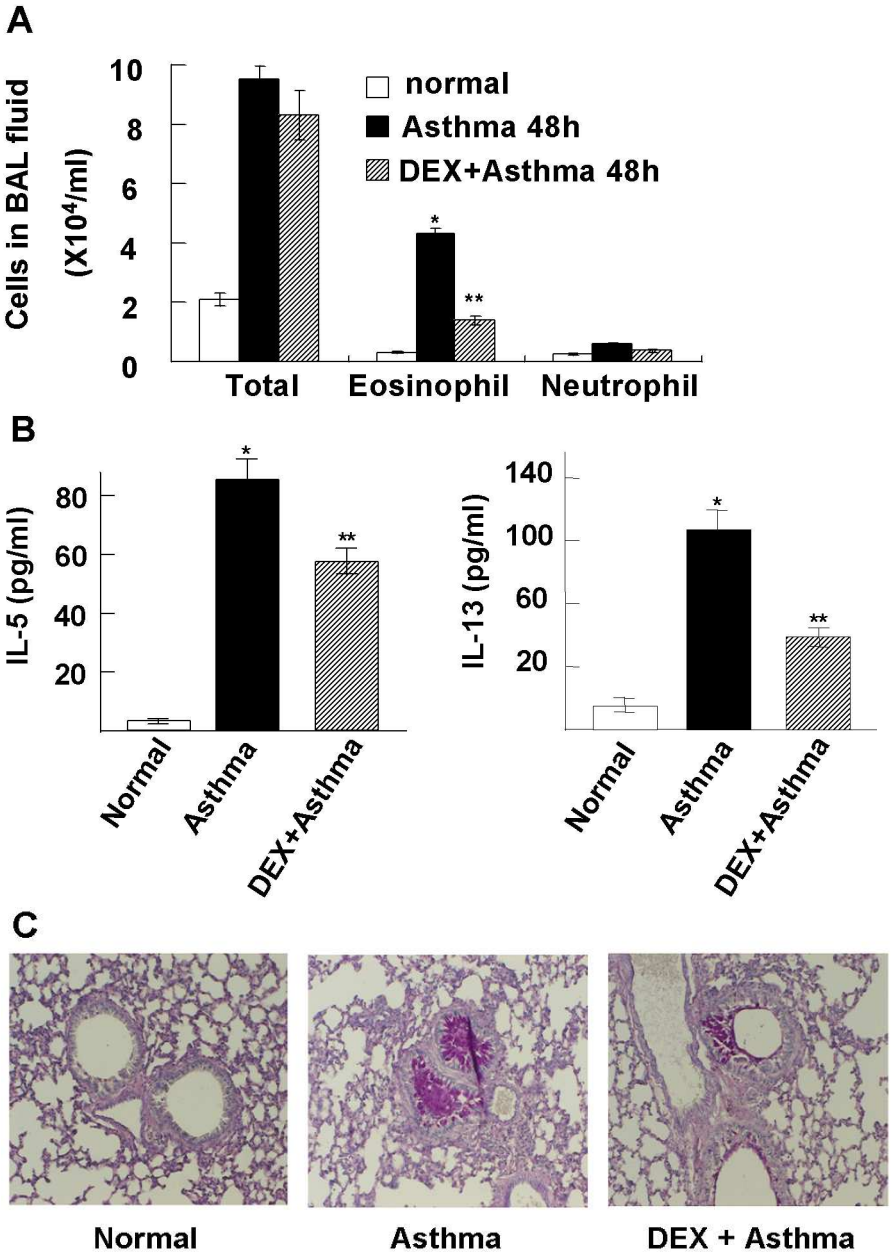

Figure 5. DEX inhibits Th2 cell-mediated asthmatic phenotypes. DEX (3.5 mg/kg) was given i.p. 1 day and 1 h prior to the second airway challenge. The numbers of BAL eosinophils (A) were assessed 48 h after the second airway challenge. IL-5 and IL-13 in BAL fluids (B) were measured by ELISA 24 h after the second airway challenge. Lungs were removed 48 h after the second airway challenge and stained with PAS (C). Results are expressed as mean ±SD of three separate experiments (n=3~5 per group). *$p<0.01$ vs. normal, **$p<0.01$ vs. asthma group.

treatment with DEX significantly suppressed the levels of each of the Th2 cytokines examined.

To determine whether DEX pretreatment also affects airway mucus production, sections of lungs were stained with PAS two days after the second OVA challenge. Numerous PAS-positive goblet cells were present in the bronchi and bronchioles of OVA-immunized and -challenged animals, but not in control mice; in some instances, bronchial lumens were filled with mucus (Fig. 5C). In contrast, the number of mucus-containing epithelial cells in the airways of DEX-pretreated mice appeared to be markedly reduced, and little or no mucus was present in the bronchial lumens.

Discussion

In this study, we investigated the manner by which DEX regulates IgG-IC-induced NF-κB activation and NF-κB-dependent asthmatic phenotypes. We found that DEX suppressed NF-κB activation, and also suppressed subsequent BAL fluid levels of TNF-α TNF-α-induced late AHR, eotaxin, and MCP-1.

However, DEX failed to suppress BAL levels of KC and MIP-2, as well as recruitment of neutrophils into the airways.

Based on the positions of cysteine residues in their amino terminal domains, chemokines can be divided into four groups: the C, C-C, C-X-C, and CX_3C families. These four groups act on different types of leukocytes; the C chemokines are principally chemotactic for $CD8^+$ T lymphocytes (22) and the C-C chemokines, which include MCP-1 and MIP-1α, mediate the chemotaxis of monocytes/macrophages but not neutrophils (23), whereas C-X-C chemokines such as MIP-2 (24), KC (25), and cytokine-induced neutrophil chemoattractant (CINC)-2 (26) act as potent chemoattractants for neutrophils but not mononuclear cells and the CX_3C chemokines mediate leukocyte migration and adhesion to endothelial cells (27). Therefore, our data clearly indicate that no inhibition of airway neutrophilia in DEX-pretreated mice is attributed to the failure of DEX to suppress the production of CXC chemokines.

The neutrophil may be an important inflammatory cell that contributes to the pathophysiology of severe asthma, since increased neutrophilic inflammation has been reported in induced sputum and in the bronchial submucosa of such patients (28-30). Increased neutrophilic inflammation has also been observed under other asthmatic circumstances, such as in patients who have died during a sudden-onset attack and in patients ventilated following an acute severe exacerbation (31,32). In addition, increased neutrophils have been observed in airway submucosal glands in patients who have died of asthma (33).

The effects of steroids on neutrophil recruitment and function remain controversial. GCs exert inhibitory effects on neutrophil activation and functions such as chemotaxis, free radical generation, and adhesion (34-37). Wilson et al. (38) demonstrated no change in submucosal IL-8 immunoreactivity after 8 weeks of ICS treatment. In contrast, GCs also inhibit neutrophil apoptosis (39-41). Additionally, GCs have been reported to have no effect on neutrophilia (42) or to even promote it (43-46) in patients with asthma in which GCs effectively reduce eotaxin expression and lung eosinophilia. This has been point of controversy during the last decade, and the mechanisms involved in such neutrophilia remain unknown. Chemokines, which are small cytokines that are involved in the recruitment of cells to a site of inflammation, might play an important role in this process. The question of whether corticosteroids show a differential regulation of eosinophil- or neutrophil-associated chemokines or whether they are pansuppressors of all chemokines in asthmatic patients, particularly in those patients with moderate-to-severe forms of the disease, remains unanswered.

The promoter regions of MIP-2 and KC also contain NF-κB binding sites, and their gene transcriptions are required for the activation of NF-κB (47,48). In addition, it has been shown that TNF-α-induced neutrophil activation and infiltration are mediated through the induction and release of CXC chemokines (49-52). However, despite the significant inhibition of NF-κB activity and TNF-α level in the BAL fluid in DEX-pretreated mice, CXC chemokine levels were not found to be inhibited by DEX. These findings suggest that a transcriptional regulatory pathway other than the NF-κB pathway acts to control CXC chemokine production, which is not inhibited by DEX. Therefore, delineation of the underlying mechanism of CXC chemokine expression may provide a clue for understanding the role of neutrophilia in patients with steroid-resistant asthma.

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