

IMMUNOPATHOGENESIS OF ASTHMA

IgG Immune Complex Induces the Recruitment of Inflammatory Cells into the Airway and TNF-Mediated Late Airway Hyperresponsiveness via NF- κ B Activation in Mice

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Background. Many of the inflammatory proteins that are expressed in asthmatic airways are regulated, at least partially, by nuclear factor (NF)- κ B. Blockade of NF- κ B activity has resulted in attenuation of the cardinal features of asthma. Thus, delineating the mechanisms involved in NF- κ B activation in asthma might provide an interesting approach to improving the management of asthma. However, despite its importance, the mechanism for NF- κ B activation in asthma has not yet been determined. **Objective.** To examine the role of IgE and IgG antibodies (Abs) in the activation of NF- κ B in mouse lungs. **Methods.** To examine the effect of IgE, mice underwent intratracheal (i.t.) instillation of an IgE immune complex (IgE-IC) (anti-2,4-dinitrophenyl hapten (DNP) IgE + DNP-BSA or DNP-OVA) and anaphylactogenic anti-IgE (LO-ME-2). For IgG, mice underwent i.t. instillation with a complex of anti-chicken gamma globulin (CGG) IgG1 mAb + CGG. NF- κ B activation was determined by gel shift assay. Small interfering RNA was used for blockade of p50 expression. The effect of tumor necrosis factor (TNF) blockade was determined using anti-TNF Ab. A previously established murine model of asthma was used to assess airway hyperresponsiveness (AHR). **Results.** A single i.t. instillation of either IgE-IC or LO-ME-2 failed to induce activation of NF- κ B in the lungs. In contrast, single i.t. instillation of IgG-IC was capable of inducing NF- κ B activation, as well as NF- κ B-dependent proinflammatory molecules, such as TNF and CXC chemokines. Pretreatment of p50 small interfering RNA decreased bronchoalveolar lavage fluid levels of TNF and macrophage inflammatory protein-2 induced by IgG-IC instillation. Single i.t. instillation of IgG-IC caused the recruitment of neutrophils and macrophages into the airway and TNF-mediated late AHR, but failed to induce Th2 cell-mediated asthmatic phenotypes. **Conclusion.** IgG, but not IgE, is the major Ab that induces not only NF- κ B activation and NF- κ B-dependent proinflammatory molecules in the lungs but also subsequent recruitment of inflammatory cells into the airway and TNF-mediated late AHR.

Keywords asthma, IgE, IgG, neutrophil, NF- κ B, TNF

INTRODUCTION

Airway inflammation is a cardinal feature of bronchial asthma. The inflammatory response in asthmatic lungs is characterized by infiltration of airway walls with mast cells, lymphocytes, and eosinophils. This cellular infiltration is associated with the increased expression of several inflammatory proteins in the airways, including cytokines, enzymes, and adhesion molecules (1). The induction of chronic cytokine expression and the recruitment and activation of inflammatory cells in asthma involve increased transcription of inflammatory cytokine genes, which are regulated by transcription factors (2).

Nuclear factor (NF)- κ B is one of the transcription factors inducing the various genes involved in immune

and inflammatory responses (3,4). Many of the inflammatory proteins that are expressed in asthmatic airways are regulated, at least in part, by NF- κ B. These include cytokines (e.g., interleukin (IL)-1 β , IL-4, IL-5, IL-9, IL-15, and tumor necrosis factor (TNF)), chemokines (e.g., RANTES, monocyte chemotactic protein-3, and eotaxin), and adhesion molecules (e.g., ICAM-1 and VCAM-1) (5–9).

Blockade of NF- κ B activity by using either mice lacking the NF- κ B subunits (10–12), or a strategy to specifically inhibit NF- κ B activity (13,14), has resulted in the attenuation of all cardinal features of asthma, including airway hyperresponsiveness (AHR), airway obstruction, and airway eosinophilic inflammation, indicating that NF- κ B is pivotal in the development of allergic airway inflammation. Thus, understanding the mechanisms of NF- κ B activation in asthma might provide an interesting approach to improving the management of asthma. However, despite its importance, the mechanisms for NF- κ B activation in asthma have not yet been determined.

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Inhalation of allergen initiates airway inflammation in part by binding of allergen to antigen-specific antibodies (Abs), which then bind to Ab receptors on innate immune cells, such as macrophages, dendritic cells, mast cells, and airway epithelial cells. As a result of these actions, NF- κ B activation probably occurs in these cells, and numerous mediators are released, which in turn initiate inflammation.

In this study, we have investigated the effects of IgE and IgG Abs on NF- κ B activation in normal mouse lungs. We have found that IgG, but not IgE, induces NF- κ B activation, resulting not only in airway neutrophilia, but also in TNF-induced late AHR.

MATERIALS AND METHODS

Materials

2,4-Dinitrophenyl hapten (DNP)-conjugated OVA and BSA were purchased from Biosearch Technologies, Inc. (Novato, CA, USA). Mouse monoclonal anti-DNP IgE was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Specific pathogen-free female C57BL/6 mice, purchased from Orient Bio Inc. (Gapyeong, Gyeonggi, Korea), were 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 under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

Intratracheal Administration

Mice were anesthetized using 80 mg/kg of pentobarbital sodium injected intraperitoneally, and placed on a vertical platform. Intratracheal (i.t.) administration was performed using a 50 μ L glass syringe (Hamilton Co., Reno, NV, USA) fitted with a 2 in, 26-gauge blunt-end needle inserted directly above the trachea.

Mast Cell Activation

Anti-DNP IgE (1 μ g) was administered i.t., followed by i.t. administration of 2 μ g of DNP-OVA or DNP-BSA 1 day later. These reagents were purchased from Sigma-Aldrich Chemicals. Anaphylactogenic anti-IgE (LO-ME-2, 1 μ g) (Zymed Laboratories Inc., San Francisco, CA, USA) was also given by i.t. administration.

IgG Immune Complex

The mouse IgG1 mAb specific for chicken gamma globulin (CGG) was prepared as ascites in pristane-primed nude mice (15). The IgG fraction was further purified using a protein G column (Sigma-Aldrich Chemicals). The IgG immune complex (IC) was prepared at twofold antigen excess, based on equivalence points determined by

quantitative precipitin curves. The IgG-IC was incubated for 2 h at 37°C and thereafter overnight at 4°C. Afterward, the IC was washed three times (500g, 10 min), resuspended in cold PBS, and stored at 4°C.

Removal of lipopolysaccharide

Detoxi-GelTM Endotoxin Removing Gel was purchased from PIERCE (Rockford, IL, USA). At first, gel and all solutions were equilibrated to room temperature. To regenerate gel, gel contained in the column was washed with five column volumes of 1% sodium deoxycholate, thereafter washed again with five column volumes of pyrogen-free water to remove detergent. OVA, IgG, and CGG were applied to the column, respectively, and then aliquots of pyrogen-free water were added and flow-through was collected finally.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed as described previously (16).

Determination of AHR

AHR was assessed as a change in airway function after airway challenge by aerosolized methacholine, as described elsewhere (16,17). After each methacholine challenge, airway resistance data were continuously collected. Maximum airway resistance values were selected to express changes in airway function, and represented a percentage change from baseline after aerosol saline challenge.

Gel Shift Assay

For the determination of NF- κ B activation in the nuclear extracts from mouse lung tissues, gel shift assays were performed as described previously (18). For supershift/inhibition assays, 1–2 μ g of specific supershifting Abs against p50, p52, p65, RelB, or c-Rel components of NF- κ B were incubated with the nuclear extract on ice for 1 h before the addition of labeled oligonucleotide to the binding reaction.

ELISA

TNF and macrophage inflammatory protein (MIP)-2 protein levels in BAL fluids were determined using ELISAs. The lower limits of detection for the cytokines were as follows: TNF assay (>5.1 pg/mL; R&D Systems) and MIP-2 assay (>1.5 pg/mL; R&D Systems) (R&D systems, Inc. 614 McKinley Place NE, Minneapolis, MN 55413 USA).

Histamine Assay

The levels of histamine in BAL were measured as described previously (18).

Treatment of Anti-TNF

Two milligrams of anti-TNF (MP6-XT22.11)⁷ and control rat IgG1, J4-1 (16), were injected i.p. 10 min before the IgG-IC instillation.

RNA Interference

Small interfering RNA (siRNA) strands for mouse NF- κ B p50 and non-targeting controls were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., 2145 Delaware Avenue, Santa Cruz, CA. 95060, USA) and Bioneer Corporation (Bioneer Corporation, 49-3, Munpyeong-dong, Daedeok-gu, Daejeon 306-220, Korea), respectively. *In vivo* siRNA transfection was performed by using *in vivo* jet polyethyleneimine purchased from Polyplus-transfection (Polyplus-transfection, BIOPARC, Boulevard Sébastien Brant, BP 90018, 67400 Illkirch, France), according to the manufacturer's instruction. p50 siRNA and polyethyleneimine dissolved in 5% glucose were mixed in a volume of 50 μ L for i.t. injection. The mixture was incubated for 15 min at room temperature and then injected 36 h before IgG-IC administration.

Immunoblotting Analysis

Immunoblotting analysis was performed to confirm that p50 siRNA blocked the expression of its target protein. Mice were killed by cervical dislocation and lungs were removed immediately and thereafter washed with cold saline and then dried with blotting paper. Nuclear extracts were obtained by using NE-PER nuclear and cytoplasmic extraction reagents purchased from Thermo Scientific (Thermo Scientific, 81 Wyman Street, Waltham, MA 02454, USA). Equal amounts of nuclear extracts were separated on a 10% SDS polyacrylamide gel under the reducing condition and transferred onto Polyvinylidene fluoride (PVDF) membranes (GE Healthcare, 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327, USA). Membranes were blocked by incubation for 1 h at room temperature in 5% skimmed milk in TBS-0.1% Tween 20 and then incubated for 2 h with primary Abs against mouse p50 and Proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology). PCNA was used as loading control. Blots were washed for 25 min with five time changes of TBS-0.1% Tween 20 solution and followed by incubation for 1.5 h with Horse radish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG Abs (Upstate (Upstate, 290 Concord Road, Billerica, MA 01821, USA) and Stressgen Bioreagents Corporation (Stressgen Bioreagents Corporation, 10 Executive Boulevard, Farmingdale, NY 11735, USA), respectively). After five times washing for 25 min, they were developed by ECL advance Western blotting detection kit (GE Healthcare).

Cell Culture

Murine alveolar macrophage cells, MH-S (ATCC CRL-2019), were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotics at 37°C in a 5% CO₂ atmosphere.

Statistical Analysis

Data were expressed as means $\pm$ SD. Statistical comparisons were performed using one-way ANOVA followed by the Fisher's exact test. Significant differences between groups were determined using the unpaired Student's *t*-test. P-value < .05 was considered statistically significant.

RESULTS

IgE Immune Complex Failed to Induce NF- κ B Activation in the Normal Mouse Lungs

We first examined whether IgE Abs induce NF- κ B activation in the normal mouse lungs. Mice underwent i.t. instillation with IgE-IC (anti-DNP IgE + DNP-BSA or DNP-OVA) or anaphylactogenic anti-IgE (LO-ME-2). Mast cell degranulating activities of these regimens were first evaluated by measuring the levels of histamine in BAL fluids. Histamine was released by the IgE-IC and LO-ME-2, but not by anti-DNP IgE, DNP-BSA, or DNP-OVA alone (Figure 1A).

Instillation of IgE-DNP-BSA complex, anti-DNP IgE alone, DNP-BSA alone, or LO-ME-2 did not induce NF- κ B activation (Figure 1B). In contrast, instillation of IgE-DNP-OVA complex resulted in NF- κ B activation. However, we unexpectedly found that NF- κ B activation also occurred in response to the negative control, DNP-OVA. The complete blocking of NF- κ B mobilization caused by the addition of the cold competitor, but not by the addition of the irrelevant motif, CRE, indicated that NF- κ B binding was specific. We further examined the effect of DNP-OVA on NF- κ B activation by i.t. instillation of varying concentrations of DNP-OVA. DNP-OVA-induced NF- κ B activation at concentrations greater than 3 μ g (Figure 1C), suggesting that OVA by itself was probably involved in this process. I.t. instillation of OVA resulted in NF- κ B activation at concentrations greater than 1 μ g (Figure 1C). OVA-induced NF- κ B activation was not caused by the contaminating lipopolysaccharide (LPS) present in commercial OVA, because removal of LPS by an affinity column did not reduce OVA activity (Figure 1C). I.t. instillation of OVA resulted in the induction of TNF and MIP-2 (Figure 1D). These data indicate that OVA by itself induces NF- κ B activation. All the data considered together indicate that IgE Abs do not seem to be involved in the activation of NF- κ B in the lungs.

IgG-IC-Induced NF- κ B Activation in the Lungs

We next examined the effect of IgG-IC on NF- κ B activation using a mixture of LPS-free anti-CGG IgG1 mAb + CGG. I.t. instillation of the IgG-IC, but neither anti-CGG IgG1 alone nor CGG alone, resulted in the activation of NF- κ B in a dose-dependent manner (Figure 2A). Supershift analysis (Figure 2B) demonstrated that addition to the supershift assay of Ab specific for the p50 subunit almost completely reduced the intensity of the DNA-binding activity, with a visible shift in the mobility of the complex. The addition of anti-p65 or anti-c-Rel Abs resulted in reduced intensity and a weaker shift in

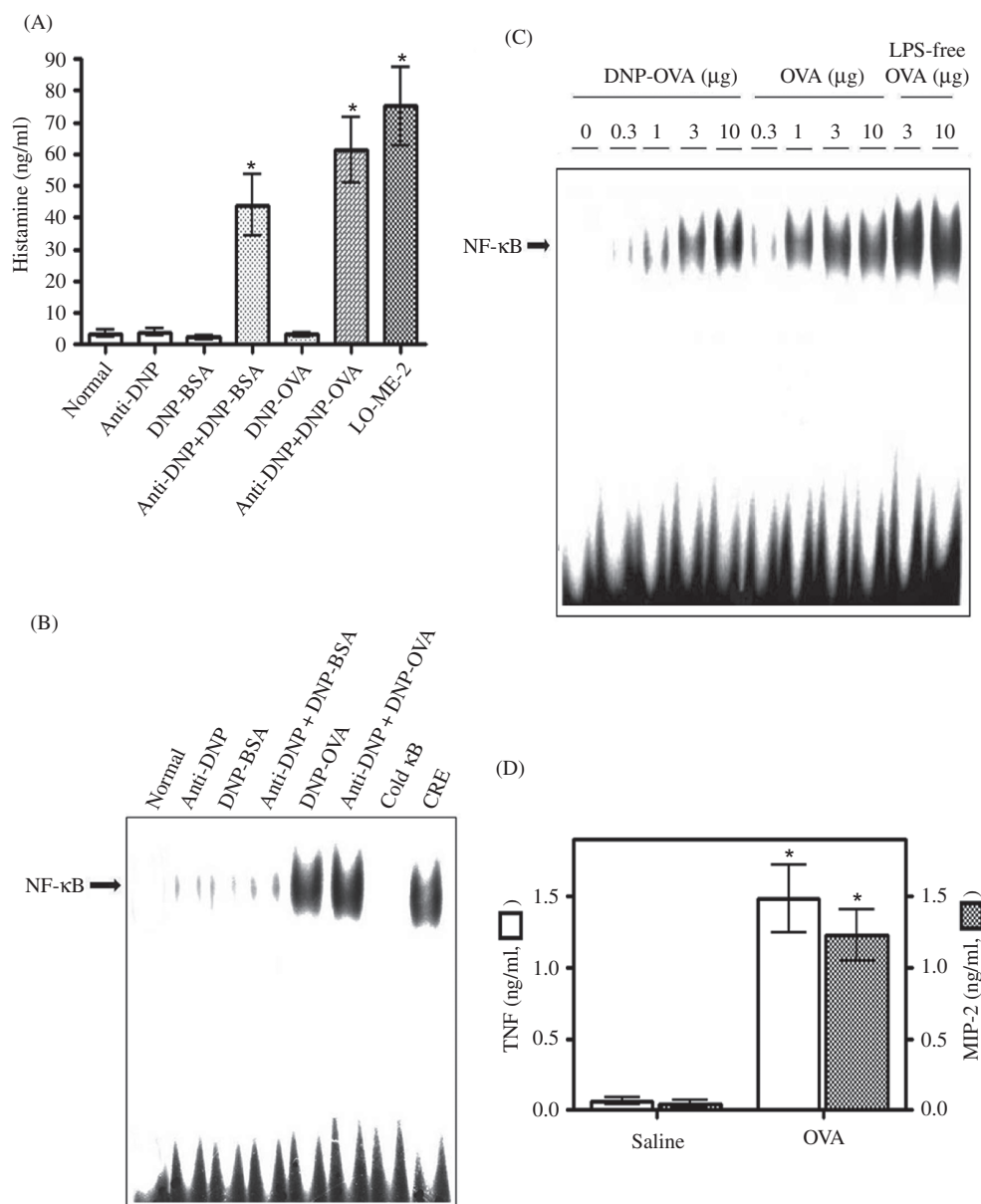


FIGURE 1.—IgE/mast cells are not involved in NF- κ B activation. (A) Histamine levels in BAL fluid were measured 20 min after the second airway challenge (asthma) or the i.t. instillation of IgE-IC (DNP-OVA + anti-DNP or DNP-BSA-anti-DNP) or LO-ME-2. (B) Lungs were removed 1 h after various challenges as indicated, and NF- κ B activation was measured by gel shift assay of nuclear extracts. (C) NF- κ B activation in the lungs of mice administered various concentrations of DNP-OVA, OVA, and LPS-free OVA. (D) BAL fluid levels of TNF and MIP-2 measured 1.5 h after i.t. instillation of 3 μ g of OVA ($n = 3$ animals for each experimental data point). * $p < .01$ versus control.

the mobility of the complex. The NF- κ B complexes were not supershifted by Abs against p52 and RelB. These results suggest that the activation of NF- κ B species in response to IgG-IC may result in the occurrence of two heterodimer types, p50-p65 and p50-c-Rel. In addition to NF- κ B activation, IgG-IC-induced NF- κ B-dependent proinflammatory molecules, including TNF and MIP-2 (Figure 2C). We also examined the effect of IgG-IC on NF- κ B activation *in vitro* using murine alveolar macrophage cells, MH-S. Treatment of the cells with IgG-IC resulted in NF- κ B activation (Figure 2D) as well as induction of TNF and MIP-2 (Figure 2E) as seen in *in vivo* study.

To clarify the role of NF- κ B in IgG-IC-induced TNF and MIP-2, we tried to block NF- κ B activation using p50 siRNA. We first confirmed the silencing efficiency of the siRNA by demonstrating that IgG-IC-mediated induction of p50 was abrogated by i.t. administration of p50 siRNA, but not control siRNA, in a dose-dependent manner (Figure 3A and B). Mice were i.t. administered with 1 or 2 nmol of p50 or control siRNA. p50 siRNA, but not control siRNA, abrogated again the IgG-IC-mediated induction of p50 (Figure 3C). Under these conditions, IgG-IC-induced TNF and MIP-2 productions were significantly inhibited (Figure 3D).

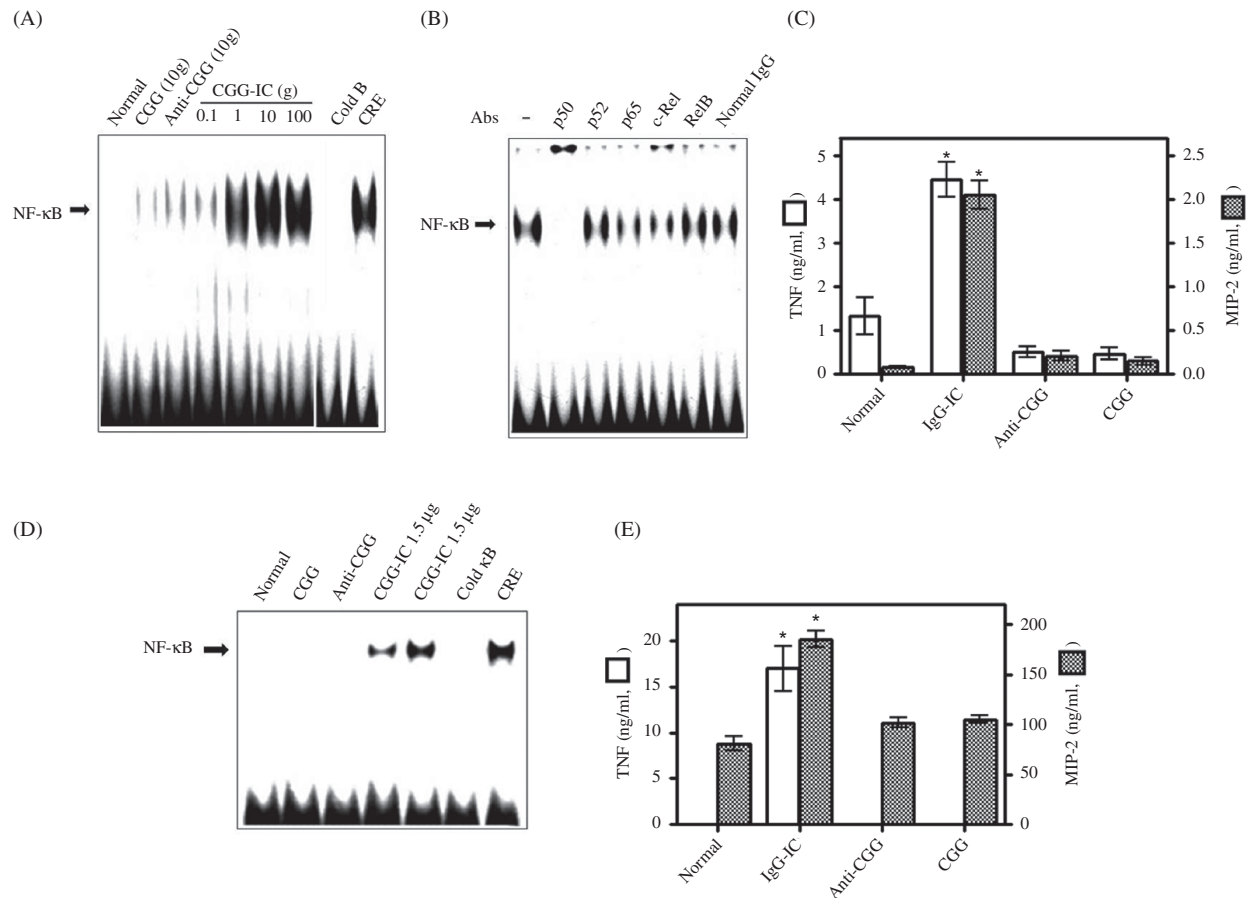


FIGURE 2.—IgG-IC induces NF- κ B activation in the lungs. (A) NF- κ B activation of lungs of mice administered various concentrations of IgG-IC. (B) Super shift assay of lungs of mice administered 10 μ g of IgG-IC. Lungs were removed 1 h after i.t. instillation of IgG-IC. (C) BAL fluid levels of TNF and MIP-2 measured 1.5 h after i.t. instillation of 10 μ g of IgG-IC or CGG or IgG. (D, E) MH-S cells were treated with IgG-IC. Supernatants and cells were collected 1.5 h after IgG-IC treatment. Data are representative of 2–5 independent experiments ($n = 3$ animals for each experimental data point). * $p < .05$ versus control.

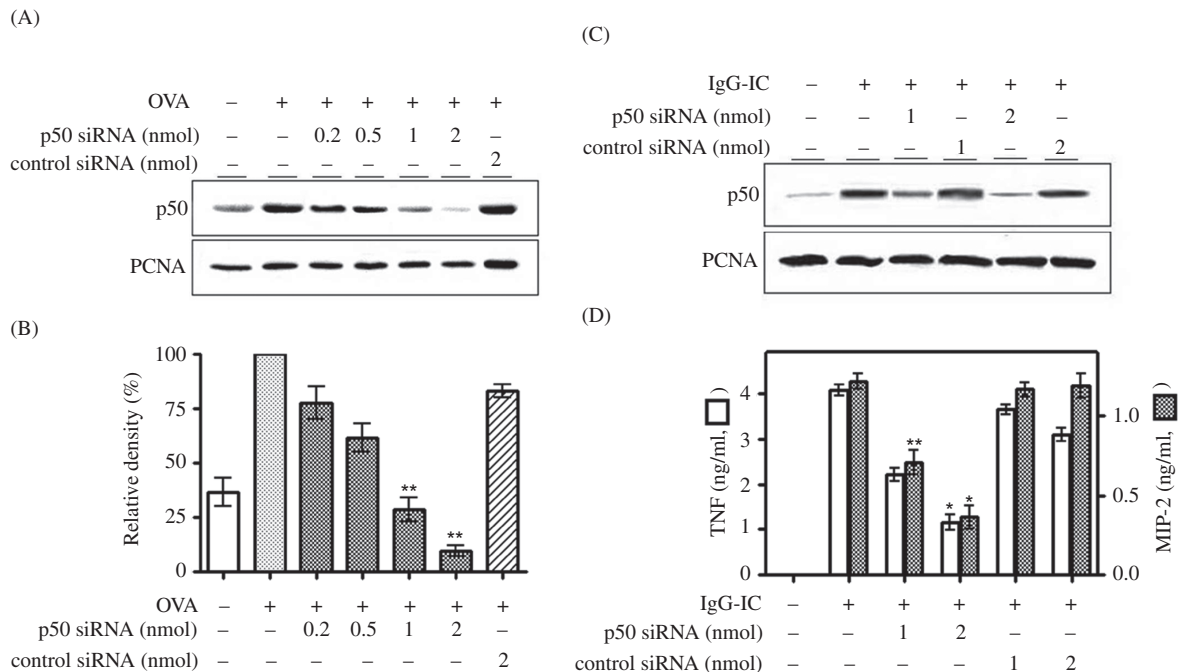


FIGURE 3.—p50 siRNA suppresses IgG-IC-induced NF- κ B activation. (A, B) Silencing kinetics of p50 siRNA. siRNA was i.t. injected with indicated dose 36 h before i.t. instillation of OVA (10 μ g). Lungs were collected 1 h after OVA instillation. p50 expression was assessed with immunoblotting analysis. Relative density was calculated as relative ratio of p50 to PCNA and data represent percentage of OVA group. (C) p50 siRNA was i.t. injected 36 h before IgG-IC instillation and lungs were collected 1 h after IgG-IC instillation. (D) BAL fluids were collected 1.5 h after IgG-IC instillation. Data are representative of 3–5 independent experiments ($n = 3$ animals for each experimental data point). * $p < .01$; ** $p < .05$ versus control.

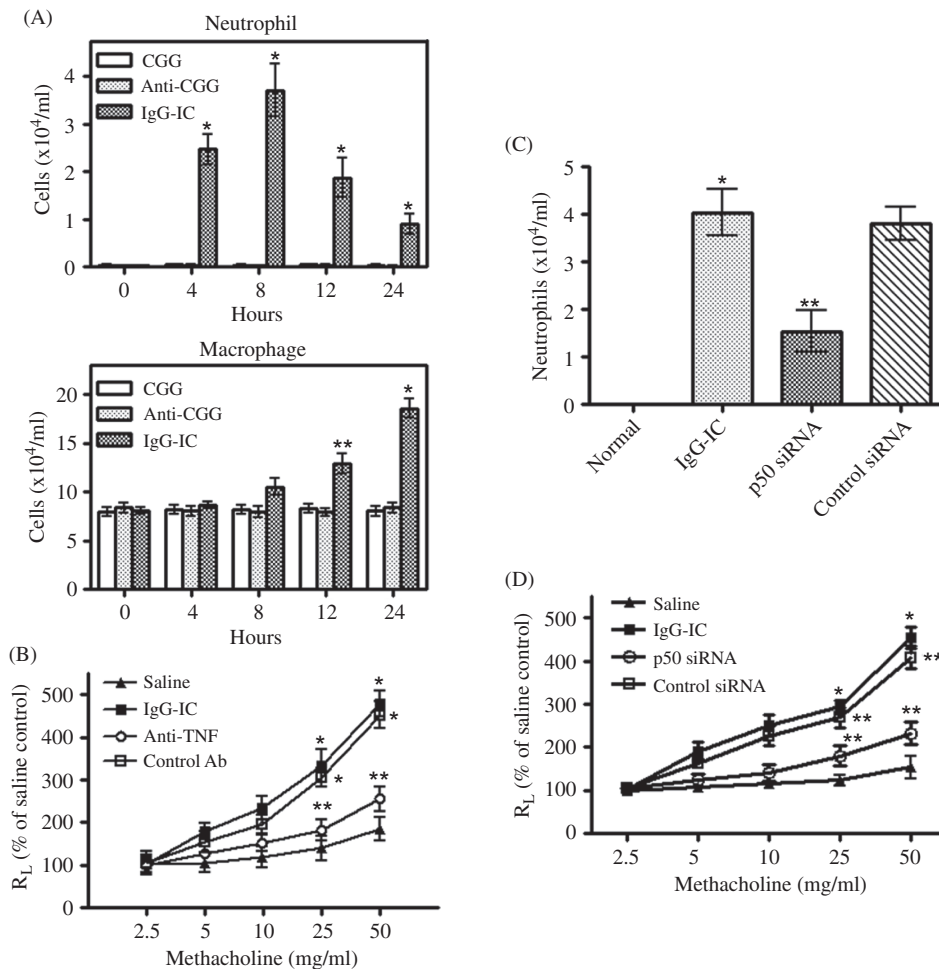


FIGURE 4.—IgG-IC induces recruitment of inflammatory cells into the airway and TNF-mediated AHR. (A) Recruitment of neutrophil and macrophages into the airway was assessed at the times indicated. Data are representative of two or three independent experiments ($n = 3$ animals for each experimental data point). * $p < .01$; ** $p < .05$ versus control. (B) Anti-TNF and control Ab were injected i.p. 10 min before the IgG-IC instillation. AHR was measured at 10 h. All mice underwent i.t. instillation of 10 μ g of IgG-IC. p50 siRNA was i.t. injected 36 h before IgG-IC instillation, the number of neutrophils in the BAL fluid (C) and AHR (D) were measured 8 h and 10 h after IgG-IC instillation, respectively. Data are representative of two or three independent experiments ($n = 3$ animals for each experimental data point). * $p < .01$ versus saline group; ** $p < .05$ versus IgG-IC.

IgG-IC-Induced Inflammatory Cell Recruitment into the Airway and Late AHR

We examined the effects of a single i.t. instillation of IgG-IC on the development of asthmatic phenotypes, including recruitment of inflammatory cells into the airways, late AHR, and Th2 cell-dependent asthmatic phenotypes such as Th2 cytokine levels in BAL fluids and airway eosinophilia. Instillation of IgG-IC resulted in the recruitment of neutrophils and macrophages from approximately 6–12 h and 12–24 h, respectively, after instillation, but it did not significantly affect the recruitment of lymphocytes (Figure 4A). Additionally, IgG-IC induced the development of late AHR measured at 10 h, but there was no further development of AHR beyond this time point, that is, at 24 and 48 h (data not shown). IgG-IC did not induce any Th2 cell-dependent asthmatic phenotypes such as increased levels of Th2 cytokines in BAL fluid and mucus secretion (data not shown).

We have recently described TNF-induced late AHR in a murine model of asthma (16,19). According to

that protocol, the reaction occurs approximately 10 h after the second allergen airway challenge. Pretreating the animals with anti-TNF, but not control Ab, before the instillation of IgG-IC abrogated the IgG-IC-induced AHR, suggesting that this AHR was caused by TNF (Figure 4B). We finally examined the role of NF- κ B in IgG-IC-induced airway neutrophilia and late AHR. I.t. administration of 2 nmol of p50, but not control siRNA, resulted in significant inhibition of IgG-IC-mediated airway neutrophilia (Figure 4C) and late AHR (Figure 4D).

DISCUSSION

In this study, we investigated the effects of IgE and IgG Abs on NF- κ B activation in mouse lungs. We have demonstrated here that IgG-IC was able to induce NF- κ B activation. This resulted in the expression of various NF- κ B-dependent proinflammatory molecules. IgG-IC-induced NF- κ B activation was not caused by contaminating LPS present in anti-CGG Ab and CGG, because (1) we removed LPS from anti-CGG Ab and

CGG by an affinity column, and (2) anti-CGG Ab or CGG alone failed to induce NF- κ B activation. Up until lately, very little was known about the role of IgG in the pathogenesis of asthma. A relationship between IgG-IC-induced NF- κ B activation and pulmonary inflammation was suggested by several investigators using a rat model of acute lung injury. In this rat lung model, intrapulmonary deposition of IgG-ICs resulted in NF- κ B activation in lung tissue, which in turn produced early proinflammatory cytokines such as TNF and IL-1 (19,20). Additionally, in an experimental model of immune glomerulonephritis, IgG-IC induced glomerular injury by mechanisms that involved NF- κ B activation in glomeruli and infiltrating cells (21,22). This information suggests that IgG-IC functions *in vivo* as an NF- κ B inducer, which supports the hypothesis that IgG-IC has an important role in the development of lung inflammation in asthmatic lungs through NF- κ B activation.

We initially thought that IgE was the candidate for NF- κ B inducer, because mast cells express the cell-surface receptor Fc ϵ RI, which binds IgE Abs with high specificity and affinity (23), and antigen-mediated cross-linkage of Fc ϵ RI triggers mast cell release of a wide spectrum of mast cell-derived preformed mediators, including platelet-activating factor (24–26) and leukotrienes (27,28), which have been reported to induce NF- κ B activation. However, we found that IgE-IC (IgE-DNP-BSA) and anaphylactogenic anti-IgE, all of which were capable of inducing not only elevated histamine levels in BAL fluid, but also passive cutaneous anaphylaxis (data not shown), did not induce NF- κ B activation. However, we have found that i.t. instillation of the IgE-IC (IgE-DNP-BSA) resulted in TNF production in mouse airways (data not shown). This suggests that IgE-IC can stimulate mast cells, resulting in NF- κ B activation in these cells. Despite this, the reason why IgE-IC failed to induce NF- κ B activation in this study was probably due to the fact that NF- κ B activation only in the mast cell population was apparently not detected by the gel shift assay of whole lung lysate, because mouse lung contains very low numbers of mast cells (29). In contrast, we can detect NF- κ B activity from the lungs of mice administered IgG-IC, because IgG-IC apparently activates NF- κ B in mast cells as well as in other inflammatory cells such as macrophages, eosinophils, and platelets, which express IgG Fc receptors on their cell surfaces (30, 31). Other authors (32) showed that IgE-ICs significantly augmented the levels of IL-4 and eosinophilia in BAL fluid of asthmatic mice. Thus, we cannot exclude the possibility that both IgE-IC and IgG-IC could induce lung injury and NF- κ B activation via distinct mechanisms and target cells.

In the course of this study, we unexpectedly found that OVA by itself induced NF- κ B activation. I.t. instillation of OVA as low as 1 g induced NF- κ B activation. Although a relationship between OVA and NF- κ B activation has not yet been reported, Taube et al. (33) have reported that stimulation of macrophages from mice not sensitized to

OVA showed a significant increase in NF- κ B-dependent cytokines, such as TNF and MIP-2, further supporting the hypothesis that OVA induces NF- κ B activation.

An asthmatic feature induced by single i.t. instillation of IgG-IC was inflammatory cell recruitment into the airway, such as neutrophils and macrophages. There is accumulating evidence that neutrophils are a component of inflammation in asthma, particularly in patients with severe asthma, since increased neutrophilic inflammation measured in induced sputum and in the bronchial submucosa has been reported in such patients (34–36). The significance of neutrophilic inflammation is that this inflammation appears to be largely resistant to the anti-inflammatory effects of corticosteroids (37–39). In this study, we demonstrated that dexamethasone, which suppressed IgG-IC-induced NF- κ B activation and elevated CC chemokine BAL levels, failed to suppress CXC chemokines and airway neutrophilia (data not shown), suggesting that neutrophilic inflammation caused by IgG-IC may be resistant to steroid treatment. Activated neutrophils are able to synthesize and release a great variety of NF- κ B-dependent proinflammatory cytokines (IL-1, IL-3, IL-6, IL-8, TNF- α , MIP) as well as other molecules (metalloproteinases, cyclooxygenase-dependent prostaglandins and thromboxanes, adhesion molecules) that play a key role in the development of inflammatory responses (40). There are several possible approaches to target NF- κ B. One promising approach is the inhibition of IKK-2 by small molecule inhibitors, several of which are now under development (41).

Another important IgG-IC-induced asthmatic feature was the development of late AHR. We demonstrated in this study that IgG-IC induced TNF-mediated late AHR. Regarding the link between TNF and late AHR, we have recently described TNF-induced late AHR occurring 8–14 h after allergen airway challenge in a murine asthma model (42). Furthermore, i.t. instillation of TNF resulted in a single peak of AHR occurring approximately 10 h after instillation (42). We demonstrated in this study that anti-TNF blocked IgG-IC-induced AHR. Collectively, these findings indicate that IgG-IC is capable of inducing late AHR via TNF production.

The initial events incurred after a challenge by IC include activation of two codominant effector pathways responsible for the subsequent development of IC inflammation: receptors for IgG Fc portion (Fc γ Rs) on resident effector cells (43) and complement system (44). Among Fc γ Rs, IC-induced hypersensitivity type III reactions in lung pathology and other immunologic diseases are mediated by the activating Fc γ RIII (45) and controlled by the inhibitory Fc γ RII (46). Regarding the signal transduction in Fc γ R-mediated NF- κ B activation, it has been reported that activation of tyrosine kinases and mitogen-activated protein kinase is required for the process in monocyte cell line (47). Recently, Rittirsch et al. (48) reported that toll-like receptor 4 and Fc γ RIII pathways are structurally and functionally connected at the receptor level, suggesting that toll-like receptor 4 plays a role in IgG-IC-mediated NF- κ B activation.

Recently, a member of the G-protein-coupled receptor proteinase-activated receptor-2, and the C-type lectin dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin-related 1 have been reported to induce NF- κ B activation (49,50) in allergic diseases (51,52), although the role of IgG-IC in the circumstances remains to be established. Further studies are required to delineate the precise mechanisms underlying IgG-IC-induced NF- κ B activation.

One of the complement activation products, C5a, has been demonstrated to be required for the full development of IgG-induced pulmonary inflammation (53). Together with other cytokines/chemokines and vascular adhesion molecules, C5a is required for recruiting leukocytes from the vascular space into the interstitial and distal airway compartments (54,55). C5a also directly activates neutrophils and macrophages for chemokine production. Both *in vitro* and *in vivo* studies have shown that, in the presence of IgG-IC, C5a, and membrane attack complex, C5b-9 cause synergistic intrapulmonary generation of rat CXC and CC chemokines, resulting in increased neutrophil accumulation as well as intensified lung injury (56, 57). Regarding complement-dependent IgG-IC-induced AHR, Lukacs et al. (58) reported that IgG-IC induces AHR to a methacholine challenge, which was most severe by 1 h, was still apparent but diminishing by 4 h, and had resolved by 24 h. These results are in contrast to our finding in terms of the occurrence of late AHR. These different results very likely reflect differences in the measurement time: our measurements were made around 10–14 h after IgG-IC instillation, while the previous group did not make at the time point.

It is generally accepted that cytokines secreted by Th2 cells seem to play a critical role in the development of late AHR, although it has often been proposed that CD4⁺ T cells promote the development of AHR indirectly through the recruitment of eosinophils and/or other leukocytes (59,60). However, the causal relationship between TNF-mediated and Th2 cell-mediated late AHR is largely unknown. Our recent unpublished data have demonstrated that in the murine model of asthma described in this study, late AHR occurs in a biphasic fashion with the first phases at 8–14 h and the second phase at 24–48 h. These two phases are caused by TNF and Th2 cells, respectively, and the former takes place independent of the latter (manuscript in preparation). This suggests that TNF is a key cytokine in the development of late AHR. In this regard, this study demonstrated for the first time that IgG plays a key role in the development of late AHR via the production of TNF.

The clinical significance of these new findings largely remains to be established. However, given that (1) high-affinity receptors for IgG (Fc γ RI) are expressed on human mast cells, macrophages, and neutrophils, and that aggregation of Fc γ RI results in the release of proinflammatory mediators including TNF (61,62); (2) inhalation of TNF in healthy (63) or asthmatic (64) human subjects leads to AHR; and (3) anti-TNF therapy in patients with asthma markedly improved asthma

symptoms, lung function, and AHR, and the most striking feature of anti-TNF therapy was the improvement in AHR (65,66), it is plausible that a similar mechanism may operate in human asthma.

In conclusion, our study provides evidence that IgG-IC failed to induce any Th2 cell-mediated asthmatic phenotypes, but was able to induce NF- κ B activation in mouse lungs, resulting in airway inflammation and TNF-induced late AHR. This suggests that the IgG/NF- κ B pathway forms an important axis in the development of allergic airway inflammation independent of dendritic/Th2 cells. Therefore, elucidation of the mechanisms underlying IgG-IC-mediated NF- κ B activation may represent a novel approach for blocking allergic airway inflammation.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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