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NF-κB Activation during IgG Immune Complex-Induced Lung Injury

Requirements for TNF-α and IL-1β but Not Complement

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The development of acute lung inflammatory injury induced by alveolar deposition of IgG immune complexes in rats requires increased production of the proinflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) as well as the complement activation product, C5a. Transcription of TNF-α and IL-1β genes are known to be regulated by the nuclear factor-kappa B (NF-κB). During IgG immune complex-induced lung inflammation, NF-κB has been shown to be activated in both alveolar macrophages and whole lung tissues. In the current studies we sought to determine whether TNF-α, IL-1β, the complement system and oxidants contribute to the activation of NF-κB in the lung. Electrophoretic mobility shift analysis of nuclear extracts from whole lung tissues demonstrated that NF-κB activation induced by the presence of IgG immune complexes occurred independently of the complement system and neutrophils. Intrapulmonary instillation of TNF-α or IL-1β into normal lung induced NF-κB, whereas C5a was incapable of causing NF-κB activation. In alveolar macrophages stimulated *in vitro* with IgG immune complexes, NF-κB activation was greatly attenuated in the presence of antibodies to TNF-α or IL-1β. Similarly, *in vivo* blockade of TNF-α or IL-1β suppressed lung NF-κB activation during IgG immune complex-induced lung injury. *N*-acetylcysteine, but not catalase, suppressed activation of lung NF-κB. These data suggest that TNF-α and IL-1β function in an autocrine or paracrine manner to amplify the lung inflammatory response through activation of NF-κB. Oxidants not derived from neutrophils also appear to play a role in this process, whereas complement activation products are not involved in this phenomenon. *(Am J Pathol 1998, 152:1327–1336)*

The transcription factor, nuclear factor-kappa B (NF-κB), regulates the expression of many early response cytokines and adhesion molecules known to contribute to the development of acute lung inflammation. The primary form of NF-κB consists of a heterodimer of NF-κB1 (p50) and RelA (p65), which in most cells is retained in the cytoplasm complexed with inhibitory proteins of the IκB family, including IκBα.[1] In response to inflammatory stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and reactive oxygen species, IκBα is phosphorylated, ubiquinated, and degraded in a process requiring the 26S proteasome.[2] Degradation of IκBα unmasks the nuclear localization sequence of NF-κB subunits, allowing translocation to the nucleus where NF-κB binds specific promoter elements and induces gene transcription.

In a rat model of acute inflammatory lung injury, deposition of IgG immune complexes causes distal airway activation of complement and generation of the complement activation product, C5a. Intrapulmonary blockade of C5a with antibody reduces lung production of TNF-α, reducing up-regulation of vascular intercellular adhesion molecule-1 (ICAM-1), and diminishing neutrophil accumulation and intensity of lung injury.[3] In this lung inflammatory response, enhanced production of TNF-α and IL-1β by activated macrophages is required for up-regulation of the adhesion molecules, ICAM-1, and E-selectin on pulmonary endothelial cells.[4,5] Interactions of these vascular adhesion molecules with their respective ligands on blood neutrophils causes leukocyte adhesion to the endothelium and recruitment of neutrophils into the alveolar compartment. The ensuing lung injury is mediated by oxidants and proteases released by neutrophils and lung macrophages and is characterized by increased vascular permeability and alveolar hemorrhage.[6]

Recent studies in this lung model show that NF-κB is rapidly activated in alveolar macrophages following IgG immune complex deposition and that activation of NF-κB follows a time course that is similar to production of TNF-α and IL-1β, recruitment of neutrophils, and development

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of lung injury.[7] However, the specific events leading to NF-κB activation during lung inflammatory injury are largely unknown. In the current studies, we investigated the roles of complement activation products, oxidants, and TNF-α and IL-1β in the induction of NF-κB activation in alveolar macrophages and in whole lung extracts during IgG immune complex-induced lung injury. Our findings demonstrate that TNF-α and IL-1β are required for NF-κB activation induced by IgG immune complexes *in vitro* and *in vivo*. Oxidants not derived from neutrophils also seem to be involved in this process whereas the complement system and C5a appear not to be required for activation of NF-κB.

Materials and Methods

Reagents

Recombinant murine TNF-α and IL-1β were purchased from R&D Systems, Inc. (Minneapolis, MN). Human C5a was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Rabbit polyclonal IgG anti-rat C5a, rabbit polyclonal IgG antimurine TNF-α, and goat polyclonal IgG anti-murine IL-1β were produced and purified as previously described.[3,8] Recombinant murine IL-1 receptor antagonist protein (IRAP) was a kind gift of Dr. Steven Chensue (Department of Pathology, University of Michigan Medical School). Rabbit polyclonal IgG anti-bovine serum albumin (BSA) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Catalase and *N*-acetylcysteine (NAC) were purchased from Sigma Chemical Co. (St. Louis, MO).

IgG Immune Complex-Induced Alveolitis

Pathogen-free male Long-Evans rats (275 to 300 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with ketamine HCl (150 mg/kg, intraperitoneally). A total of 1.5 mg of anti-BSA in a volume of 0.3 ml of phosphate-buffered saline (PBS) was instilled via an intratracheal catheter during inspiration. Immediately thereafter, 10 mg of BSA (<1 ng endotoxin/mg) in 0.5 ml of PBS (pH 7.4) was injected intravenously. Control rats received PBS intratracheally and BSA intravenously. For intervention studies, anti-C5a (400 μg), anti-TNF-α (300 μg), anti-IL-1β (500 μg), NAC (5 mg), or catalase (5 mg) were administered intratracheally together with the IgG anti-BSA. At the indicated time points, rats were exsanguinated and the pulmonary circulation flushed with 10 ml of saline by pulmonary artery injection. The lungs were surgically dissected and immediately frozen in liquid nitrogen.

Lung Instillation of C5a, TNF-α, or IL-1β

Rats received intratracheal instillations of PBS, C5a (2.5 μg), TNF-α (200 ng), or IL-1β (50 ng) in 0.3 ml of PBS. At the indicated time points, rats were exsanguinated and the pulmonary circulation flushed with 10 ml of saline by pulmonary artery injection. The lungs were surgically dissected and immediately frozen in liquid nitrogen. For rats receiving PBS or C5a, bronchoalveolar lavage (BAL) fluids were collected and TNF-α content was measured using a standard WEHI cell cytotoxicity assay as previously described.[9]

Complement and Neutrophil Depletion Studies

Complement depletion in rats was accomplished by intraperitoneal injection of 25 units of purified cobra venom factor at 36, 24, and 12 hours before the induction of IgG immune complex-induced lung injury. This methodology reduces plasma C3 levels to less than 3% of their original levels.[10] Neutrophil depletion was achieved by intraperitoneal injection of a rabbit anti-rat neutrophil antibody (0.5 ml; Accurate Chemical & Scientific Corp., Westburg, NY) 16 hours before pulmonary IgG immune complex deposition. This technique reduces blood neutrophil counts by >95% (to <200 PMN/mm^3 blood).

Alveolar Macrophages

Alveolar macrophages from normal rat lungs were isolated by BAL. Alveolar macrophages (1×10^6 cells/ml) in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) were plated in 100-mm dishes and allowed to adhere for 1 hour in a humidified incubator at 37°C with 5% CO_2. IgG-BSA immune complexes were produced by slowly adding 4 volumes of IgG anti-BSA (5 mg/ml) to 1 volume of BSA (10 mg/ml) and incubating for 30 minutes at 37°C. IgG-BSA immune complexes were recovered by centrifugation and resuspended in Dulbecco's modified Eagle's medium. Alveolar macrophages were stimulated with PBS or IgG-BSA immune complexes (100 μg/ml) in the absence or presence of anti-TNF-α (100 μg/ml) or IRAP (5 μg/ml).

Nuclear Protein Extraction

Nuclear extracts of whole lung tissues were prepared by the method of Deryckere and Gannon.[11] Briefly, frozen lungs were homogenized in solution 1 (0.6% Nonidet P-40, 150 mmol/L NaCl, 10 mmol/L HEPES, pH 7.9, 1 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, and 1 μg/ml pepstatin). The homogenate was incubated on ice for 5 minutes and then centrifuged for 5 minutes at 5000 rpm at 4°C. The supernatant was discarded, and proteins from the pelleted nuclei were extracted by incubation on ice for 30 minutes with 2 pellet-volumes of solution 2 (25% glycerol, 20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 1.2 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, and 1 μg/ml pepstatin). Nuclear extracts of alveolar macrophages were prepared using methods based on those described by Dignam et al.[12] Briefly, cells (5×10^6 to 1×10^7) were harvested by scraping and centrifuged at 1200

rpm for 5 minutes at 4°C. The supernatant was removed and the cell pellet was transferred to a 1.5-ml microfuge tube and washed twice with 1 ml of ice-cold buffer A (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol) containing a protease inhibitor cocktail (1 μmol/L aprotonin, 1 μmol/L leupeptin, 1 μmol/L pepstatin, 0.5 mmol/L phenylmethylsulfonyl fluoride). The cell pellet was resuspended in 3 pellet-volumes of buffer A plus 0.1% Nonidet P-40 and incubated on ice for 5 minutes prior to centrifugation at 14,000 rpm for 10 minutes at 4°C. Nuclear proteins were extracted from the pellet with 2 pellet-volumes of buffer C (20 mmol/L HEPES, pH 7.9, 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, 25% glycerol) containing the protease inhibitor cocktail on ice for 15 minutes. Following centrifugation at 14,000 rpm, the supernatant containing the nuclear proteins was removed and diluted 1:3 with buffer D (20 mmol/L HEPES, pH 7.9, 50 mmol/L KCl, 0.5 mmol/L dithiothreitol, 20% glycerol) containing the protease inhibitor cocktail. Protein concentrations were determined by bicinchoninic acid assay with trichloroacetic acid precipitation using BSA as a reference standard (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded NF-κB consensus oligonucleotide (5′-GTGAGGGGACTTTCCCAGGC-3′; Promega, Madison, WI) was end-labeled with γ[^{32}P]ATP (3,000 Ci/mmol at 10 mCi/ml; Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of protein (10 μg), and 35 fmols (~50,000 cpm, Cherenkov counting) of oligonucleotide were performed for 30 minutes in binding buffer (4% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, pH 8.0, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris, pH 7.6, 50 μg/ml poly(dI-dC); Pharmacia, Piscataway, NJ). For supershift analyses, antibodies to p50, p52 (NF-κB2), p65, p68 (RelB), or p75 (c-Rel) (all antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA) were added 15 minutes after the addition of radiolabeled NF-κB oligonucleotide. Reaction volumes were held constant to 15 μl. Reaction products were separated in a 4% polyacrylimide gel in 0.25× TBE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) and analyzed by autoradiography. NF-κB activation was quantitated from digitized autoradiography films using image analysis software (Adobe Systems, San Jose, CA).

Western Blot Analysis

Whole lungs were homogenized in solution 1, sonicated, and centrifuged at 5000 rpm. Interfering IgG anti-BSA in homogenate supernatant was removed with Gammabind G sepharose (Pharmacia, Piscataway, NJ). Protein concentrations were determined as described for nuclear extracts. Samples were separated in a denaturing 10% polyacrylimide gel and transferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with TBS (40 mmol/L Tris, pH 7.6, 300 mmol/L NaCl) containing 5% nonfat dry milk for 12 hours at 4°C. Membranes were then incubated in a 1:1000 dilution of rabbit polyclonal anti-IκBα (Santa Cruz Biotechnology) in TBS with 0.1% Tween 20 (TBST). After 3 washes in TBST, membranes were incubated in a 1:50,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL). Immunoreactive proteins were detected by enhanced chemiluminescence.

Statistical Analyses

All values are expressed as mean ± SEM. Data were analyzed with a one-way analysis of variance with subsequent multiple comparisons made using the Tukey test. Differences were considered significant when $P < 0.05$. For calculations of percent change, negative control values were subtracted from positive control and treatment group values.

Results

Time Course for NF-κB Activation and Lack of Neutrophil Requirement for Induction of Lung NF-κB during IgG Immune Complex-Induced Alveolitis

The time course of NF-κB activation during lung inflammatory injury was established by EMSA of whole lung nuclear extracts at various time points following alveolar deposition of IgG immune complexes. Increases in nuclear translocation (activation) of NF-κB occurred within 2 hours but with greater activation 4 hours after initiation of injury (Figure 1A). Progressive lung NF-κB activation correlated with disappearance of the inhibitory protein IκBα in lung tissues, which was greatly reduced 4 hours after IgG immune complex deposition (Figure 1B). DNA-binding competition experiments using a 50-fold excess of unlabeled NF-κB oligonucleotides identified NF-κB-specific and nonspecific banding patterns in EMSA blots (Figure 2). Because lung NF-κB activation increased in a manner consistent with the recruitment of neutrophils and because neutrophil-derived oxidants might be a causative factor in NF-κB activation, we investigated the effects of neutrophil depletion on IgG immune complex-induced NF-κB activation in whole lungs. Depletion of neutrophils with rabbit anti-neutrophil serum had no measurable effect on lung NF-κB activation (Figure 3A). These results suggest that development of IgG immune complex-induced NF-κB activation is neither caused by oxidants released from sequestered neutrophils nor an artifact because of NF-κB activation in accumulating neutrophils.

Absence of a Complement Requirement for Lung NF-κB Activation

To investigate potential contributions of the complement system in nuclear translocation of NF-κB induced by

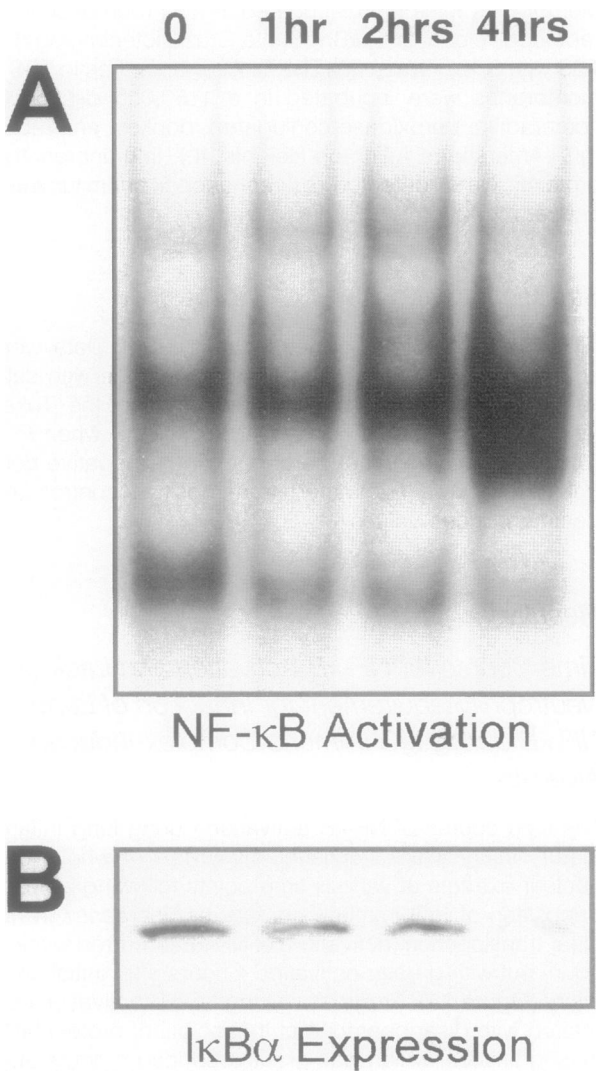

Figure 1. Time course of NF-κB activation (**A**) and IκBα protein expression (**B**) during IgG immune complex-induced alveolitis. Nuclear extracts from whole lung tissues were subjected to EMSA analysis of NF-κB activation (**A**). Whole lung homogenates were analyzed for IκBα protein by Western blot (**B**). Results are representative of three separate time-course experiments.

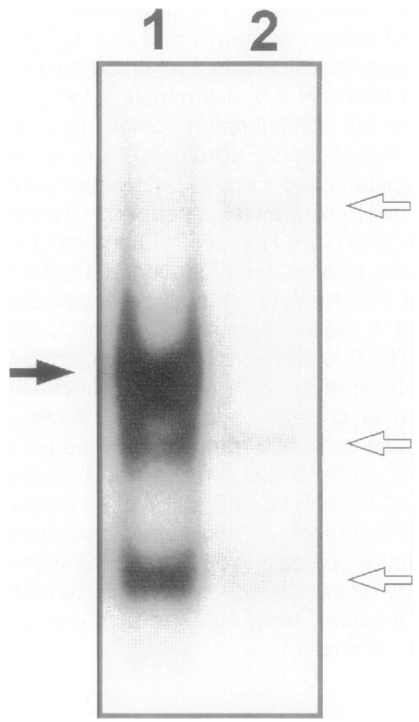

Figure 2. Specificity of the NF-κB oligonucleotide probe. DNA-binding reactions with nuclear extracts from whole lungs harvested 4 hours after IgG immune complex deposition were incubated with ^{32}P-labeled NF-κB oligonucleotide (**lane 1**). Specificity of the NF-κB oligonucleotide was confirmed by competition experiments using the same nuclear extracts incubated with a 50-fold excess of unlabeled NF-κB oligonucleotide (**lane 2**). These experiments identified NF-κB-specific (**solid arrow**) and nonspecific (**open arrows**) banding patterns.

alveolar IgG immune complex deposition, rats were depleted of complement by serial intraperitoneal injections of cobra venom factor. Because pulmonary NF-κB activation was greatest 4 hours after IgG immune complex deposition (Figure 1), this time point was chosen to determine if the complement system was required for NF-κB activation. Electrophoretic mobility shift analysis of lung nuclear extracts showed that systemic depletion of complement did not reduce lung NF-κB activation induced by IgG immune complex deposition (Figure 3B). Neutralization of C5a in the alveolar compartment by intratracheally administered anti-C5a antibodies profoundly suppressed IgG immune complex-induced lung injury in association with reduced accumulation of neutrophils.[3] Therefore, we assessed whether the protective effects of C5a blockade might be related to inhibition of NF-κB activation in the lung. Intratracheal administration of anti-C5a had no effect on lung NF-κB activation induced by IgG immune complex deposition (Figure 4A). To further investigate whether C5a may contribute to pulmonary NF-κB activation, human C5a was administered intratracheally in the absence of anti-BSA. C5a by itself did not induce NF-κB activation in lung tissues (Figure 4B) nor did it increase TNF-α content in BAL fluids (PBS, 233.4 ± 17.8 pg/ml; C5a, 156.7 ± 21.1 pg/ml; n = 4 for each group).

In Vitro *Autocrine/Paracrine Roles of TNF-α and IL-1β for Alveolar Macrophage NF-κB Activation*

Because activation of alveolar macrophages (including nuclear translocation of NF-κB) occurs in the early phase (0.5 hour) of lung inflammation[7] and because the production of TNF-α and IL-1β by alveolar macrophages appears to drive the lung inflammatory response, we assessed the roles of these cytokines *in vitro* using IgG immune complex-induced NF-κB activation. Alveolar macrophages from normal rats were stimulated *in vitro* with preformed IgG immune complexes in the presence or absence of anti-TNF-α antibody or IRAP. In PBS-treated cells, little NF-κB was present in cell nuclei (Figure 5A). However, in alveolar macrophages stimulated with IgG immune complexes, NF-κB translocation was observed after 30 minutes of stimulation (Figure 5B). In the presence of either anti-TNF-α or IRAP (Figure 5, C and D), nuclear translocation of NF-κB was attenuated. In

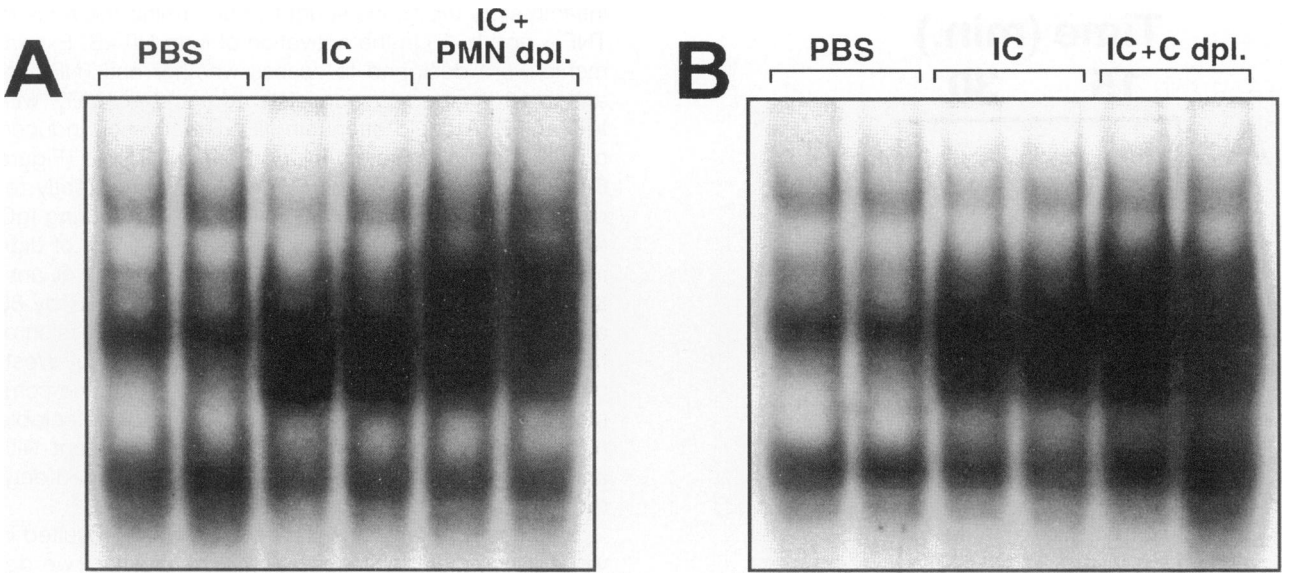

Figure 3. Effects of neutrophil depletion (**A**) or complement depletion (**B**) on IgG immune complex-induced lung NF-κB activation. NF-κB activation in whole lung tissues harvested 4 hours after intratracheal administration of PBS or IgG immune complexes (**IC**) (anti-BSA followed by intravenous infusion of BSA). Rats were either depleted of neutrophils (**PMN dpl.**) or complement (**C dpl.**) prior to IgG immune complex deposition. Results shown are from duplicate experiments.

order to quantitate NF-κB activation, digitized EMSA blots were subjected to computer-aided image analysis. Treatment with anti-TNF-α or IRAP reduced NF-κB activation by 41 and 44%, respectively. Thus, it appears that TNF-α and IL-1β produced by alveolar macrophages in response to IgG immune complexes serve to further activate alveolar macrophage NF-κB.

In Vivo *Requirement for TNF-α and IL-1β in Lung NF-κB Activation*

It is well established that TNF-α and IL-1β are potent stimulators of NF-κB activation *in vitro*. Because both of these early response cytokines are known to play important roles in the IgG immune complex model of lung

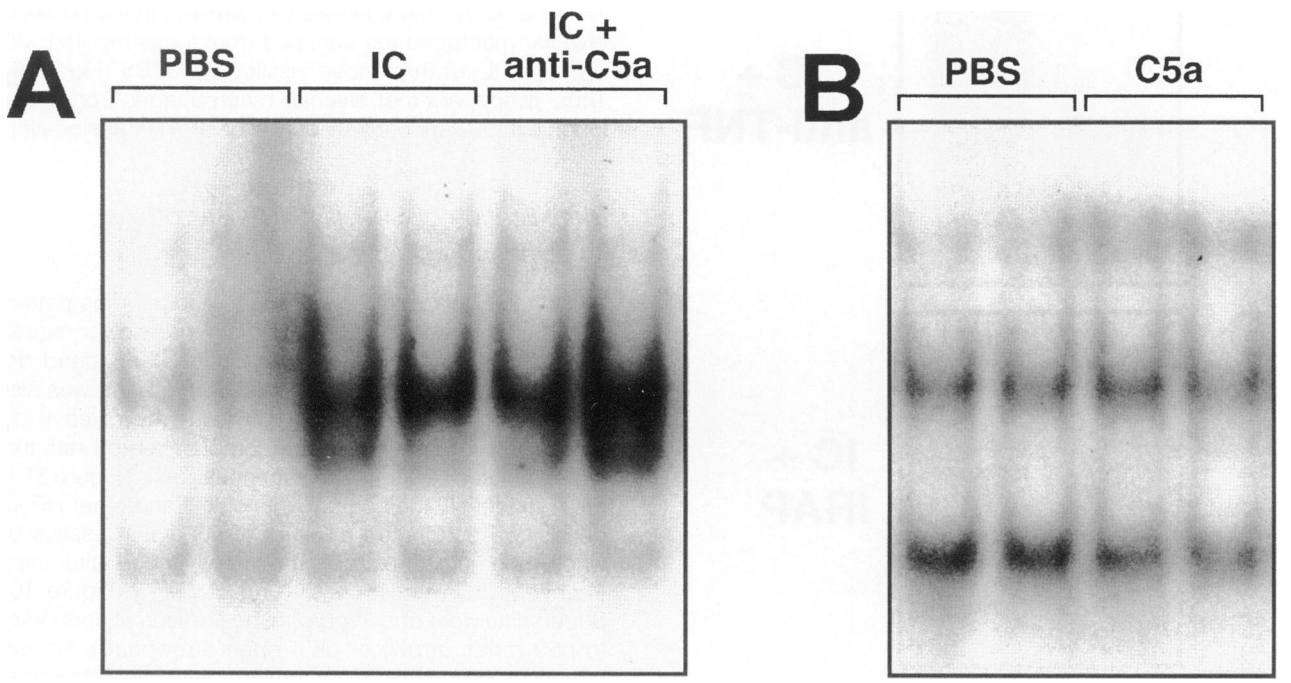

Figure 4. Effects of intrapulmonary C5a-blockade on IgG immune complex-induced lung NF-κB activation (**A**). NF-κB activation in whole lung tissues harvested 4 hours after intratracheal administration of PBS or IgG immune complexes (**IC**) (anti-BSA followed by intravenous infusion of BSA). Some rats receiving IC also received 400 μg of rabbit polyclonal anti-C5a with the anti-BSA (IC + anti-C5a). Results shown are representative of 4 separate experiments. Effects of C5a on NF-κB activation in lung (**B**). NF-κB activation in whole lung tissues harvested 4 hours after lung instillation of PBS or 2.5 μg of human C5a. Results shown are representative of 4 separate experiments.

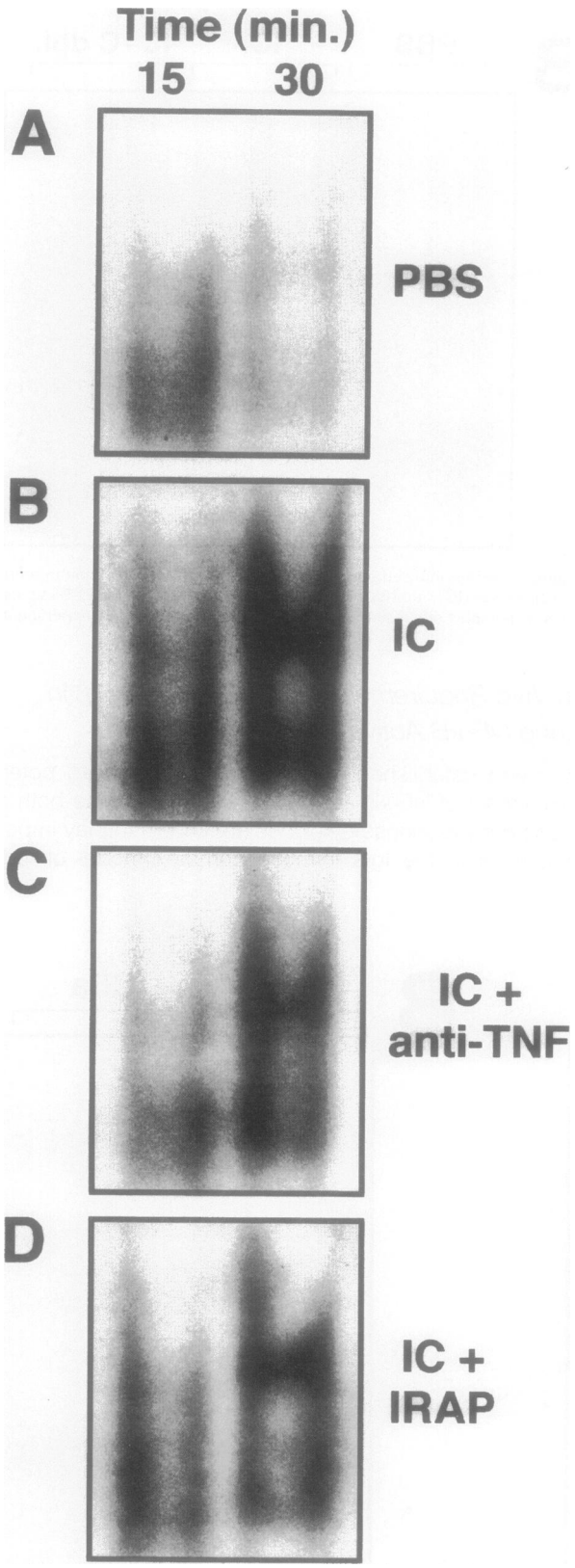

Figure 5. Effects of TNF-α or IL-1β blockade on NF-κB activation induced *in vitro* in IgG immune complex (IC) stimulated alveolar macrophages. Alveolar macrophages harvested by BAL were stimulated with PBS (**A**), 100 μg/ml IgG IC (**B**), IC + 100 μg/ml anti-TNF-α (**C**), or IC + 5 μg/ml murine IRAP (**D**).

inflammatory injury, we sought to determine the roles of TNF-α and IL-1β in the activation of lung NF-κB. Experiments were designed to assess whether anti-TNF-α or anti-IL-1β antibodies administered intratracheally with IgG anti-BSA could attenuate NF-κB activation induced during lung inflammatory injury. Both anti-TNF-α (Figure 6A) and anti-IL-1β (Figure 6B) cotreatments greatly reduced nuclear translocation of NF-κB in lung during IgG immune complex-induced injury. Image analysis of digitized EMSA blots demonstrated that anti-TNF-α or anti-IL-1β reduced nuclear localization of lung NF-κB by 80 and 81%, respectively ($P < 0.01$). Protein expression of IκBα in whole lung homogenates was measured by Western blot. Intrapulmonary deposition of IgG immune complexes caused a decrease in the amount of detectable IκBα protein in lung tissue (Figure 7). Treatment with anti-TNF-α inhibited IgG immune complex-induced degradation of IκBα protein in lung homogenates.

Because blockade of either TNF-α or IL-1β resulted in virtually complete inhibition of NF-κB activation, we determined whether exogenous administration of these cytokines could activate lung NF-κB *in vivo* in the absence of the inflammatory reactions induced by IgG immune complexes. For these experiments, either TNF-α or IL-1β (in the absence of IgG anti-BSA) was instilled intratracheally. Lung instillation of TNF-α caused increases in lung NF-κB activation similar to that of IgG immune complex deposition, with increases seen 2 hours after administration and greater increases observed after 4 hours (Figure 8A). IL-1β had similar effects (Figure 8B). To determine whether alveolar macrophages demonstrated a similar pattern of NF-κB activation, alveolar macrophages were obtained by BAL 30 minutes after lung instillation of either TNF-α or IL-1β. More NF-κB was present in the nuclei of alveolar macrophages obtained from lungs instilled with TNF-α or IL-1β than those instilled with PBS (Figure 9). Thus, it appears that alveolar macrophages from those lungs exhibit very early evidence of NF-κB translocation.

Identification of Specific NF-κB Proteins Involved in EMSA Analyses

In rats receiving intratracheal TNF-α, the banding pattern of NF-κB included a prominent band in a slower migrating position than that for the specific NF-κB band described in Figure 2. The slower migrating band was also observed at the 1-hour time point in rats given IL-1β (Figure 8) and in IgG immune complex-treated rats that were depleted of complement or neutrophils (Figure 3). In order to identify the components of this and other NF-κB bands, we conducted a series of supershift assays on whole lung nuclear extracts harvested 4 hours after intratracheal administration of 200 ng of TNF-α (Figure 10). Supershifts were observed with the addition of antibodies to p50 (open arrow) or p65 (open arrowhead). No evidence of supershifts were present with the addition of antibodies to p52 (NF-κB2), p68 (RelB), or p75 (c-Rel) proteins, suggesting that these proteins are not significant components of the NF-κB complex. The intensity of the primary NF-κB band (solid arrow) was reduced in

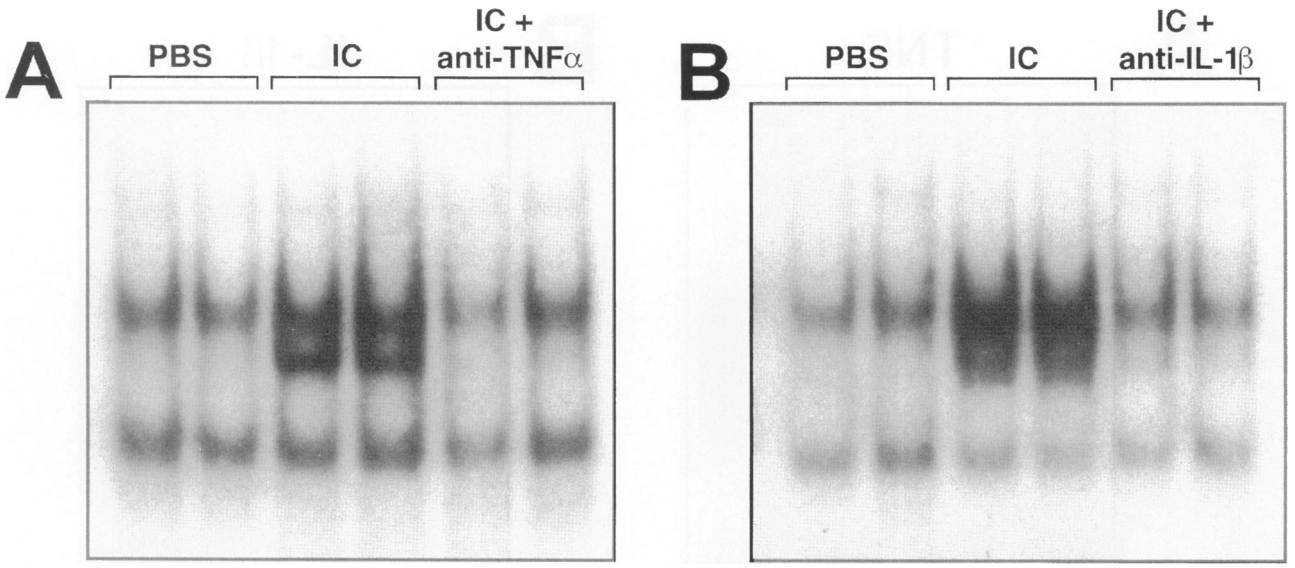

Figure 6. Effects of TNF-α blockade (**A**) and IL-1β blockade (**B**) on IgG immune complex-induced lung NF-κB activation. NF-κB activation in whole lung tissues harvested 4 hours after intratracheal administration of PBS or IgG IC (anti-BSA followed by intravenous infusion of BSA). Some rats receiving IC also received rabbit polyclonal anti-TNF-α (300 μg) or polyclonal goat anti-IL-1β (500 μg) with the anti-BSA. Results shown are representative of five (**A**) or four (**B**) separate experiments. Image analysis of digitized EMSA blots (in relative intensity units): **panel A** (n = 5/group), PBS, 86.3 ± 5.3; IC, 146.9 ± 10.3; IC + anti-TNF-α, 98.7 ± 16.7 ($P < 0.01$ *versus* IC); **panel B** (n = 4/group), PBS, 113.8 ± 7.5; IC, 190.4 ± 2.0; IC + anti-IL-1β, 129.4 ± 5.0 ($P < 0.01$ *versus* IC).

supershift assays with addition of antibody to p65 and, to a lesser extent, in the presence of antibody to p50, indicating that this band was composed primarily of p65 and p50 proteins. The slower migrating band (solid circle) all but disappeared with the supershift of p65, indicating that it was composed predominantly of p65 protein.

Role for Oxidants in Lung NF-κB Activation

Since we have shown that IgG immune complex deposition in lung mediates activation of NF-κB via TNF-α and IL-1β (Figures 5, 6, and 8), and *in vitro* studies have shown that TNF-α and IL-1β activate NF-κB through the generation of reactive oxygen intermediates,[13] we assessed whether treatment with antioxidants could reduce IgG immune complex-induced lung NF-κB activation. Administration of the thiol-based antioxidant NAC, which was given intratracheally with IgG anti-BSA, greatly suppressed lung NF-κB activation (Figure 11). Image analysis of digitized EMSA blots indicated that NAC reduced nuclear localization of lung NF-κB by 68% ($P < 0.01$).

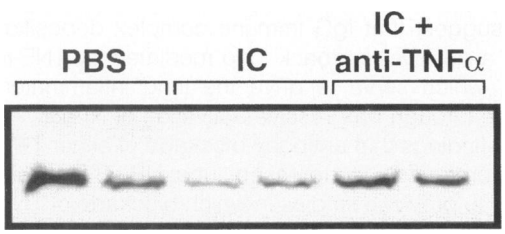

Figure 7. Effects of TNF-α blockade on pulmonary IκBα protein expression during IgG immune complex-induced lung inflammation. Western blot analysis of whole lung homogenates obtained 4 hours after intratracheal administration of PBS or IgG IC (anti-BSA followed by intravenous infusion of BSA). Some rats receiving IC also received 300 μg of rabbit polyclonal anti-TNF-α with the anti-BSA. Results are representative of three separate experiments.

However, cotreatment with catalase (administered intratracheally with anti-BSA) did not reduce lung NF-κB activation, and *in vitro* experiments showed that catalase did not reduce NF-κB activation in alveolar macrophages stimulated with TNF-α (data not shown).

Discussion

The stimulation of NF-κB by inflammatory mediators has been extensively studied using cell cultures. Cell activation by TNF-α or IL-1β causes rapid degradation of the regulatory protein IκBα, allowing nuclear translocation of the NF-κB complex, which results in transcriptional activation of target genes.[2] Similarly, it has been shown recently that the complement membrane attack complex activates NF-κB in endothelial cells to cause increased expression of cytokines, chemokines, and adhesion molecules.[14,15] However, little is known regarding the mechanisms of NF-κB regulation *in vivo*. In the present study, we investigated the roles of inflammatory mediators in activation of NF-κB during IgG immune complex-induced lung injury. In this model, BAL content of TNF-α and IL-1β as well as lung injury increases concurrently with increases in NF-κB activation.[7,8,16] It is known that TNF-α and IL-1β are required for up-regulation of ICAM-1 and E-selectin in the pulmonary vasculature because blockade of either cytokine reduces up-regulation of adhesion molecules on endothelial cells and greatly diminishes lung recruitment of neutrophils.[14,16] *In vitro* gene transcription of both ICAM-1 and E-selectin in endothelial cells is known to be regulated by NF-κB.[17] We now provide evidence that TNF-α and IL-1β may mediate this up-regulation *in vivo* by activating NF-κB, inasmuch as blockade of TNF-α or IL-1β clearly abrogated activation

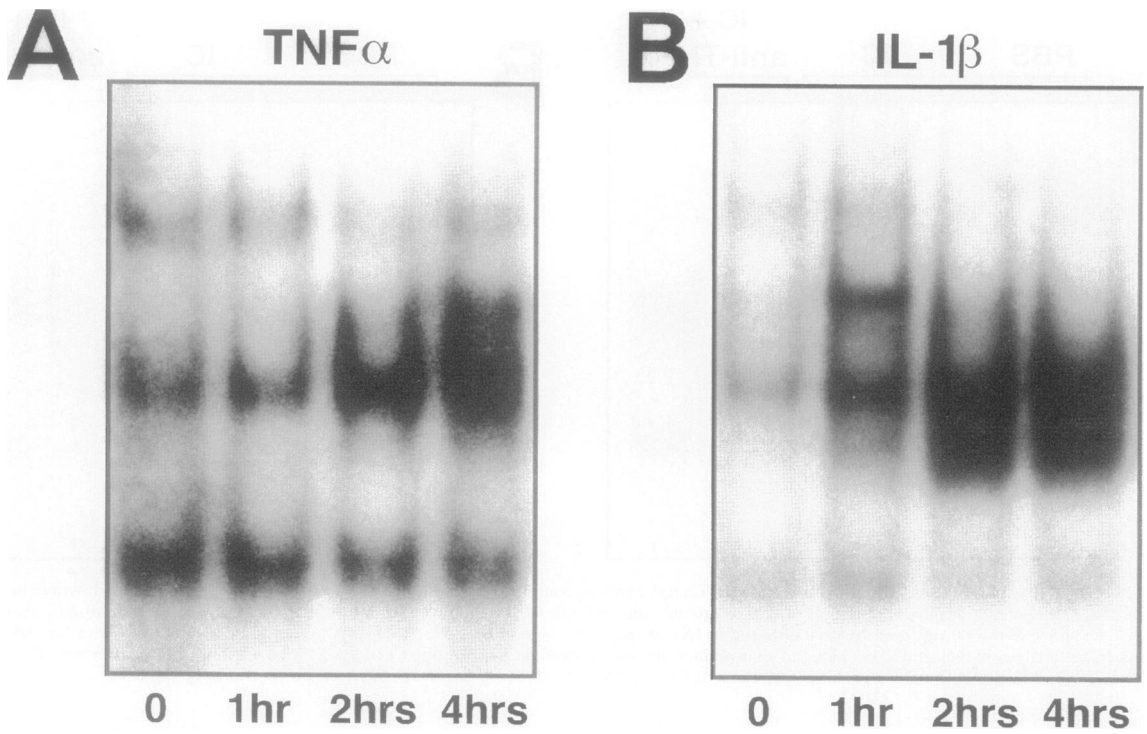

Figure 8. Temporal induction of lung NF-κB activation after instillation of 200 ng of murine TNF-α (**A**) or 50 ng of murine IL-1β (**B**). Results shown are from duplicate experiments.

of lung NF-κB following intrapulmonary deposition of IgG immune complexes (Figure 6).

Interestingly, both TNF-α and IL-1β not only activate NF-κB but are themselves induced by NF-κB,[18,19] suggesting that these cytokines may amplify inflammation through a positive feedback mechanism. Our *in vitro* studies suggest that stimulation of alveolar macrophages with IgG immune complexes results in positive feedback facilitated by production of TNF-α and IL-1β. We have demonstrated that NF-κB activation in alveolar macrophages stimulated *in vitro* with IgG immune complexes is greatly reduced by blockade of endogenous TNF-α or IL-1β (Figure 5), indicating that *in vitro* these cytokines serve to amplify cellular activation by accelerating the nuclear translocation of NF-κB. Similarly, blockade of either TNF-α or IL-1β *in vivo* resulted in almost complete inhibition of lung NF-κB activation following IgG immune complex deposition in lung (Figure 6). At least in the case of TNF-α blockade, diminished activation of NF-κB was associated with preserved expression of IκBα protein, indicating that in lung tissues, activation of NF-κB by TNF-α occurs through degradation of IκBα. In addition, lung instillation of either TNF-α or IL-1β caused progressive increases in lung NF-κB activation similar to that of IgG immune complexes (Figure 8). Taken together, the data suggest that IgG immune complex deposition initiates a positive feedback loop mediated by TNF-α and IL-1β, which serve to drive the lung inflammatory response through progressive activation of NF-κB.

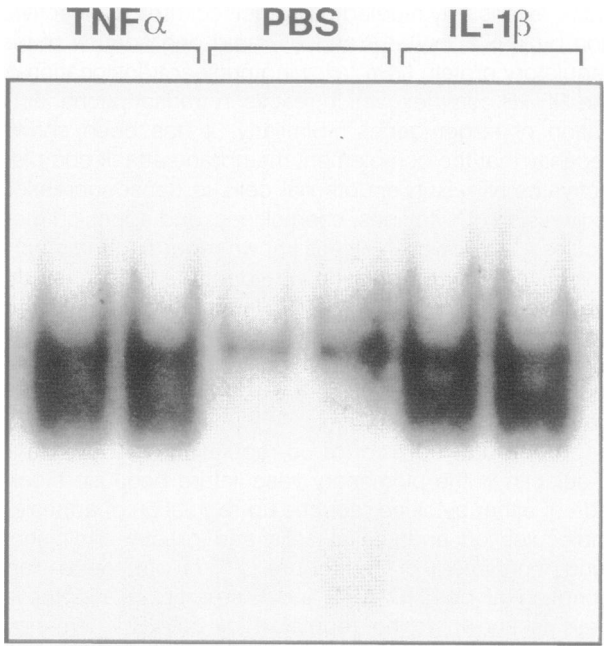

Figure 9. NF-κB activation in alveolar macrophages harvested 30 minutes after lung instillation of 200 ng of murine TNF-α, 50 ng of murine IL-1β, or PBS. Results shown are from duplicate experiments.

The findings that antibody blockade of either TNF-α or IL-1β profoundly suppressed lung NF-κB activation is similar to previous studies in which blockade of TNF-α or IL-1β resulted in dramatic reductions in lung vascular permeability and alveolar hemorrhage.[20] Synergistic interactions between TNF-α and IL-1β have been reported to cause augmentation of neutrophil recruitment and development of lung injury.[21] Thus, it appears likely that TNF-α and IL-1β synergize in the lung to cause nuclear

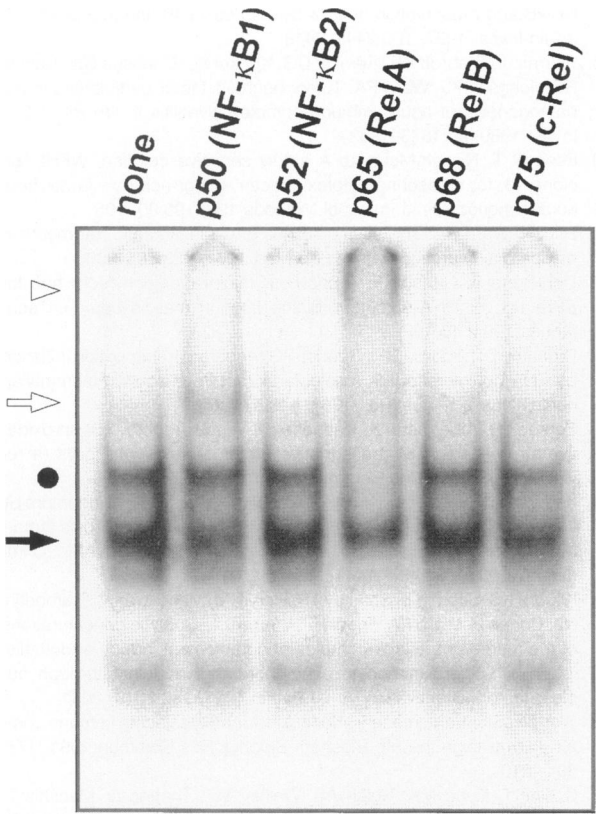

Figure 10. Supershift analysis of NF-κB components. DNA-binding reactions with nuclear extracts from whole lungs harvested 4 hours after intratracheal instillation of 200 ng of TNF-α were incubated with ^{32}P-labeled NF-κB oligonucleotide in the absence or presence of antibodies to the NF-κB proteins indicated (p50, p52, p65, p68, p75). Supershifts of p50 and p65 are indicated by an **open arrow** and an **open arrowhead**, respectively. The **solid circle** indicates the position of the high molecular weight NF-κB complex induced by TNF-α. The **solid arrow** indicates the position of the primary NF-κB band.

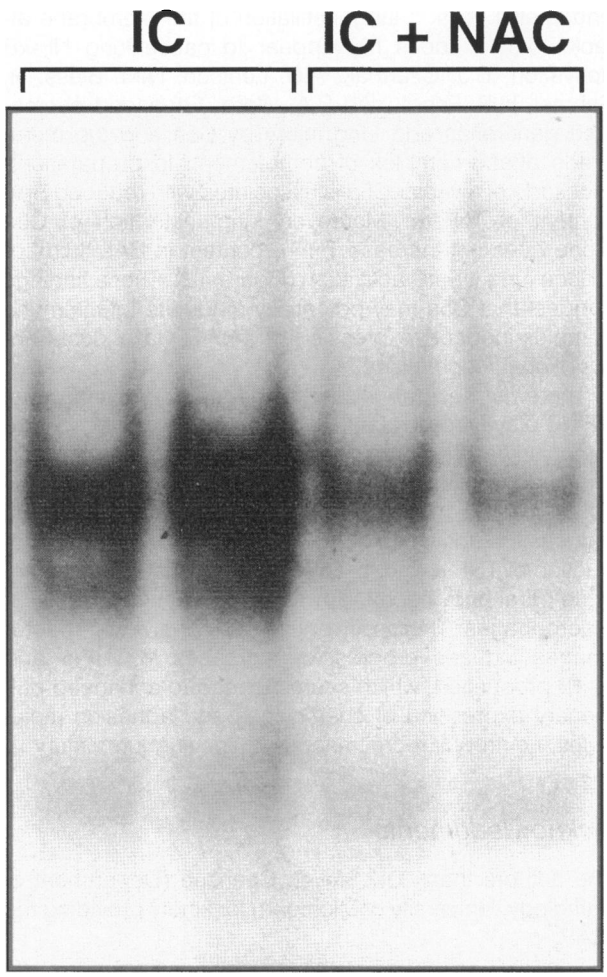

Figure 11. Effects of *N*-acetylcysteine (**NAC**) on IgG immune complex-induced lung NF-κB activation. NF-κB activation in whole lung tissues harvested 4 hours after intratracheal administration of IgG IC (anti-BSA followed by intravenous infusion of BSA). Some rats receiving IC also received 5 mg of NAC with the anti-BSA. Results shown are representative of four separate experiments. Image analysis of digitized EMSA blots (in relative intensity units): PBS, 88.3 ± 3.8; IC, 151.8 ± 5.3; IC + NAC, 108.8 ± 10.0 ($P <$ 0.01 *versus* IC); $n = 4$ for all groups.

translocation of NF-κB, inasmuch as blockade of either cytokine reduced by 80% the amount of lung NF-κB activation (Figure 6). Alternatively, TNF-α and IL-1β may function in a sequential manner. An example of sequential interactions of cytokines in the IgG immune complex model is found in studies in which macrophage inflammatory protein-1α has been blocked. Under such conditions, there is a subsequent dramatic decrease in BAL levels of TNF-α and suppression of the inflammatory response.[22] It has been concluded that lung macrophages produce macrophage inflammatory protein-1α, which functions as an autocrine stimulator of macrophages, enhancing TNF-α generation. Whether there is the same type of sequential interaction involving TNF-α and IL-1β remains to be determined.

In vitro, TNF-α and IL-1β are known to cause intracellular generation of reactive oxygen metabolites resulting in activation of NF-κB.[13] Our present data suggest that a similar oxidant-related mechanism may be intact *in vivo*. The antioxidant NAC has been shown to protect against endotoxin-induced alveolitis in a manner associated with reduced NF-κB activation.[23] While our studies using NAC in the IgG immune complex model support these suggestions (Figure 11), catalase, which scavenges H_2O_2 and protects against lung injury,[24] does not inhibit activation of lung NF-κB. Unlike NAC, which is a very small molecule (163 d) that easily crosses cell membranes, catalase is very large (~240 kd), making its passage into cells unlikely. Considering that neither the depletion of neutrophils nor catalase treatment reduced lung NF-κB activation, our data suggest that extracellular oxidants derived from neutrophils do not contribute to NF-κB activation *in vivo*. Furthermore, the suppressive effects of NAC suggest that intracellular oxidants generated in response to IgG immune complexes, TNF-α and/or IL-1β are a necessary step in the activation of lung NF-κB.

The fact that neither systemic complement depletion nor blockade of C5a had any effect on lung NF-κB activation induced by IgG immune complex deposition (Figures 3B and 4A) suggests that products of the complement cascade are not required for NF-κB activation in this model. Although the membrane attack complex of complement has been shown to activate NF-κB in cultured

endothelial cells,[15] lung instillation of the membrane attack complex does not appear to cause lung NF-κB activation (B.J. Czermak, A.B. Lentsch, N.M. Bless, H. Schmal, H.P. Friedl, and P.A. Ward: Enhanced chemokine generation and lung injury by C5a and the membrane attack complex of complement. In preparation). Blockade of alveolar C5a is associated with reduced BAL content of TNF-α.[3] Moreover, lung instillation of C5a alone does not increase TNF-α content in BAL fluids or induce lung NF-κB activation (Figure 4B). These findings suggest that C5a may potentially modulate IgG immune complex-induced expression of TNF-α via a post-transcriptional mechanism.

There is now abundant evidence suggesting that NF-κB plays a central role in the induction of lung inflammatory injury. The present studies suggest that in the IgG immune complex model of lung injury the initial stimulation of alveolar macrophages by IgG immune complexes induces the activation of NF-κB and the subsequent production of TNF-α and IL-1β, which feed back in an autocrine/paracrine mechanism to further stimulate alveolar macrophages. The positive feedback of alveolar macrophages induces progressive increases in TNF-α and IL-1β production, which seems to lead to enhanced pulmonary expression of chemokines and adhesion molecules, neutrophil recruitment, and ensuing lung injury.

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