

German Cockroach Proteases Regulate Interleukin-8 Expression via Nuclear Factor for Interleukin-6 in Human Bronchial Epithelial Cells

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German cockroach extract synergistically regulates tumor necrosis factor- α (TNF- α)-induced interleukin (IL)-8 expression in human airway epithelial cells. The IL-8 promoter contains nuclear factor (NF)- κ B, activating protein (AP)-1, and NF for IL-6 (NF-IL6) transcription factor binding regions. Because cockroach extract activates extracellular regulated kinase (ERK), a known activator of AP-1 and NF-IL6, we focused on the regulation of these transcription factors. Although TNF- α and cockroach extract both increased AP-1 translocation, mutation of the AP-1 site in the context of the wild-type promoter had no effect on cockroach extract-induced synergy. Mutation of the NF-IL6 site in the context of the wild-type IL-8 promoter, or overexpression of a dominant-negative NF-IL6 mutant, each abolished cockroach extract-induced synergy. Cockroach extract induced NF-IL6 translocation and DNA binding, an effect that was further increased in the presence of TNF- α . Cockroach extract-induced regulation of NF-IL6 was due to active serine proteases in the extract as well as activation of protease activated receptor (PAR)-2, but not PAR-1. Chemical inhibition of ERK also attenuated cockroach extract-induced NF-IL6-DNA binding. We conclude that proteases in German cockroach extract regulate PAR-2 and ERK to increase NF-IL6 activity and synergistically regulate TNF- α -induced IL-8 promoter activity in human airway epithelium.

Keywords: *Blattella germanica*; mitogen activated protein kinase; protease activated receptor-2; serine protease

Airborne allergens are known to induce asthma in predisposed individuals. Many of these airborne allergens contain protease activity, which may modulate the inflammatory response. For example, proteases in house dust mite *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* have been shown to induce proinflammatory cytokines such as interleukin (IL)-8 in cultured airway epithelial cells (1, 2). We have recently shown that German cockroach *Blattella germanica* extract synergistically increased tumor necrosis factor- α (TNF- α)-induced IL-8 expression in human bronchial epithelial (16HBE14o-) cells (3). We have also found that crude cockroach extract contains serine protease activity and this protease is responsible for mediating the synergy found in the presence of TNF- α . Cockroach proteases cleave protease activated receptor (PAR)-2 (4), resulting in an intracellular signaling cascade consisting of a G-coupled protein, Ras, MEK (mitogen activated protein (MAP) kinase/extracellular regulated kinase (ERK)), and ERK (5). In this report we investi-

gate the transcriptional mechanism by which cockroach extract synergistically regulates the IL-8 promoter.

IL-8 is a CXC chemokine (where the first two NH₂-terminal cysteines are separated by a single nonconserved amino acid) that potently attracts and activates neutrophils. Neutrophils are one of the first inflammatory cells to be recruited in response to allergen, and peripheral blood neutrophils are activated during both early and late asthmatic reactions induced by allergens (6). Both neutrophils and IL-8 are increased in patients with severe persistent asthma (7), status asthmaticus requiring mechanical ventilation (8), and sudden-onset fatal asthma (9). IL-8 is produced in response to a number of noxious or infectious agents, including lipopolysaccharide (LPS), TNF- α , and respiratory syncytial virus (RSV). The importance of IL-8 in lung inflammation is suggested by at least two studies. In the first, neutralization of the IL-8 receptor, CXCR2, in immunocompetent mice resulted in impaired neutrophil antifungal activity which resulted in lung disease and death following *Aspergillus* infection (10). In a second study, CXCR2 knockout mice failed to develop persistent airway features (i.e., persistent peribronchial inflammation, airway hyperreactivity, IgE production, Th2 profile, and airway remodeling) of chronic fungal asthma following *Aspergillus* infection (11). Together, these data suggest that the neutrophil chemoattractant ability of IL-8 is important in the pathophysiology of asthma and inflammation in the airways.

The transcriptional regulation of IL-8 expression in lung epithelial cells is complex, and involves nuclear factor (NF)- κ B, activating protein (AP)-1 and NF for IL-6 (NF-IL6) (c/EBP β) promoter sequences (12–14). TNF- α -induced transcription from the IL-8 promoter activates the classical NF- κ B pathway in the airway cell lines A549 (15) and 16HBE14o- (16). More recently, many inflammatory cytokines and chemokines, including IL-8, have been shown to be regulated by MAP kinases. MAP kinase activation increases a number of transcription factors, including the AP-1 family members c-Fos, c-Jun, and NF-IL6 (17). The requirement of ERK for IL-8 expression has been shown in A549 (18), BEAS-2B human bronchial epithelial cells (19), and 16HBE14o- cells (17). In addition, ERK has been shown to activate NF-IL6 in a variety of cells (17, 20).

In this study, we examine the precise contribution of cockroach extract to TNF- α -induced IL-8 promoter activation. We have previously shown that cockroach proteases significantly activate ERK (5). Therefore, in this article we focus on regulation of the transcription factors AP-1 and NF-IL6. We find that AP-1 is an important basal mediator of IL-8; however, activation of NF-IL6 by cockroach extract mediates the synergistic increase of TNF- α -induced IL-8 promoter activation.

MATERIALS AND METHODS

Cell Culture

An SV40-transformed human bronchial epithelial cell line (16HBE14o-), provided by S. White (University of Chicago) was studied as described previously (16). Cells were grown on coated plates

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(fibronectin, 10 $\mu\text{g/ml}$; collagen, 30 $\mu\text{g/ml}$; bovine serum albumin, 100 $\mu\text{g/ml}$) in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 200 mM of L-glutamine. Normal human bronchial epithelial cells (Clonetic Corp., Walkersville, MD) were grown according to the manufacturer specifications.

Cells were treated with human TNF- α (R&D Systems, Minneapolis, MN) in the absence or presence of whole body extract of German cockroach (*B. germanica*) purchased from Greer Laboratories (Lenoir, NC). In some experiments cells were treated with the selective PAR-2-activating peptide SLIGKV or the selective PAR-1-activating peptide TFRIFD (Invitrogen, Carlsbad, CA). In some cases, cells were pre-treated with PD98059 (25 μM) for 1 h before addition of cockroach extract and/or TNF- α .

Real-Time PCR

Cells were deprived of serum for 16 h before treatment with TNF- α (3 ng/ml), cockroach extract (10 $\mu\text{g/ml}$), or both for 4 h. RNA was extracted using a standard TRIzol method of phenol extraction. Total RNA is converted to cDNA by reverse transcriptase. IL-8 primers, designed to span an intron, are right (ATT GCA TCT GGC AAC CCT AC) and left (CTG CGC CAA CAC AGA AAT TA). IL-8 was amplified by PCR using SYBR Green on the iCycler (Bio-Rad Laboratories, Hercules, CA) as follows: 1 cycle 95°C for 3 min, followed by 35 cycles of (95°C for 5 s, 60°C for 5 s, 72°C for 10 s), 95°C for 1 min, 55°C for 1 min, and then a hold of 25°C. We chose succinate dehydrogenase complex subunit A (SDHA) as our housekeeping gene. The SDHA primers used are right (TGG GAA CAA GAG GGC ATC TG) and left (CCA CCA CTG CAT CAA ATT CAT G), and also span an intron. SDHA was amplified as follows: 1 cycle 95°C for 3 min, followed by 30 cycles of (95°C for 5 s, 60°C for 5 s, 72°C for 10 s), 95°C for 1 min, 55°C for 1 min, and then a hold of 25°C. Each target gene (IL-8) sample is normalized to a housekeeping or reference gene (SDHA) using the calculation $(E^{\text{ref}})^{C_{\text{t ref}}}/(E^{\text{tar}})^{C_{\text{t tar}}}$, where E is the real-time efficiency of the reference (ref) or target (tar) gene and C_{t} is the threshold cycle of the reference (ref) or target (tar) gene.

Plasmid Vectors

The -162/+44 fragment of the full-length human IL-8 promoter subcloned into a luciferase reporter plasmid was obtained from A. Brasier (University of Texas, Galveston) (12). Site-directed mutagenesis of the AP-1 site in the context of the -162/+44 hIL-8 promoter was introduced by polymerase chain reaction with mutagenic primers to obtain hIL-8 Δ AP-1-Luc (12). Site-directed mutagenesis of the NF-IL6 site in the context of the -162/+44 hIL-8 promoter was introduced by polymerase chain reaction with mutagenic primers to obtain hIL-8 Δ NF-IL6-Luc using the GeneTailor site-directed mutagenesis system (Invitrogen). The NF- κ B and AP-1 reporter plasmids (NF- κ B-TATA Luc, AP-1-TATA Luc, respectively) were purchased from Stratagene (La Jolla, CA). MGF-82, a myelomonocytic growth factor promoter, containing an additional 42-bp region harboring two c/EBP(NF-IL6)-binding sites was a gift from R. C. Smart (North Carolina State University, Raleigh, NC) (21). A vector containing the dominant-negative (LIP) isoform of rat c/EBP β was provided by M. S. Kilberg (University of Florida, Gainesville, FL) (22).

Transient Transfection of Human Airway Epithelial Cells

Cells were grown to 70% confluence, rinsed in Optimem (Invitrogen), and incubated with a solution of plasmid DNA (0.5 μg total DNA per 35 mm dish), Lipofectamine (4 μl /dish) and Optimem. After 5 h, the liposome solution was replaced with 10% FBS/MEM. After overnight incubation, cells were serum-starved for 8 h. Selected cells were then treated with cockroach extract (10 $\mu\text{g/ml}$), TNF- α (3 ng/ml), or both. Sixteen hours after treatment, cells were harvested and analyzed for luciferase activity using the Promega Luciferase Assay system (Promega, Madison, WI). Luciferase and β -galactosidase activity were measured as previously described (16).

Electrophoretic Mobility Shift Assay

All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were treated with cockroach extract, aprotinin-treated cockroach extract (10 $\mu\text{g/ml}$ for 15 min at 37°C before addition to cells), TNF- α , SLIGKV (50 μM), or a combination of the above for

1 h. Cells were then washed twice with PBS, harvested by scraping into 1 ml of PBS, and pelleted at 6,000 rpm for 5 min. The pellet was washed twice with PBS, resuspended in one packed cell volume of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% Nonidet P-40, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride), and incubated for 5 min with occasional vortexing. After centrifugation at 6,000 rpm, one cell pellet volume of extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 M EDTA, 1.5 mM MgCl₂, 25% [vol/vol] glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. The nuclear proteins were isolated by centrifugation at 14,000 rpm for 15 min. Protein concentrations were determined by Bradford assay (Bio-Rad) and stored at -70°C until use.

The NF-IL6 oligonucleotide probe (5'-GCCATCAGTTGCAAA TCGT-3') corresponding to the NF-IL6 site in the IL-8 promoter (Invitrogen) was used for electrophoretic mobility shift assay (EMSA). The probe was labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD) and purified in Bio-Spin chromatography columns. Four micrograms of nuclear proteins were preincubated with binding buffer containing 5 mM Tris-HCl (pH 7.5), 37.5 mM KCl, 0.5 mM EDTA, 2% Ficoll, 50 $\mu\text{g/ml}$ poly(dI-dC), and 100,000 counts/min of [γ -³²P]-labeled probe, and incubated on ice for 15 min. Cold NF-IL6 probe was added at 5 $\times$ the concentration of the radiolabeled probe. Nuclear extracts were added and the mixture was incubated at room temperature for 15 min. Protein-nucleic acid complexes were resolved with a nondenaturing polyacrylamide gel consisting of 5% acrylamide and run in 0.5 $\times$ Tris-borate-EDTA buffer (45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA) for 1 h at constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under a vacuum at 80°C for 1 h, and exposed to photographic film at -70°C with an intensifying screen.

NF-IL6 ELISA

Cells were treated as described for EMSA. Nuclear extracts were obtained using a Nuclear Extract kit (Active Motif, Carlsbad, CA). TransAM colorimetric transcription factor measurement (Active Motif) for c/EBP β was run according to manufacturers specifications. Nuclear extracts were added to a 96 well plate coated with oligonucleotide that contains a c/EBP consensus binding site (5'-GCAAT-3'). Activated c/EBP α and c/EBP β in the nuclear extract can specifically bind to this oligonucleotide. However the addition of a primary antibody which recognizes an accessible epitope against c/EBP β upon DNA binding, specifically allows for the quantification of c/EBP β . A wild-type NF-IL6 consensus oligonucleotide is used to specifically compete for binding.

Statistical Analysis

When applicable, statistical significance was assessed by one-way ANOVA. Differences identified by ANOVA were pinpointed by Student-Newman-Keul's multiple range test.

RESULTS

Cockroach Extract Synergistically Increases TNF- α -Induced IL-8 mRNA

We have previously shown that cockroach extract synergistically increased TNF- α -induced IL-8 expression in 16HBE14o- human bronchial epithelial cell line and in primary human bronchial epithelial cells (3, 5). Quantitative real-time PCR was performed in both cell types and confirmed that cockroach extract synergistically increased TNF- α -induced IL-8 mRNA levels (Figure 1).

Regulation of AP-1 by TNF- α and Cockroach Extract

To test the requirement of the AP-1 transcription factor binding site on cockroach extract-induced synergy of the IL-8 promoter, we transiently transfected cells with either the wild-type IL-8 promoter or a promoter construct with a mutation of the AP-1 site. Mutation of the AP-1 binding site significantly decreased basal activity (65% decrease in promoter activity), indicating that the AP-1 site is a basal-level enhancer (Figure 2A). In addition, mutation of the AP-1 binding site resulted in a diminished response to TNF- α . However, cockroach extract was still

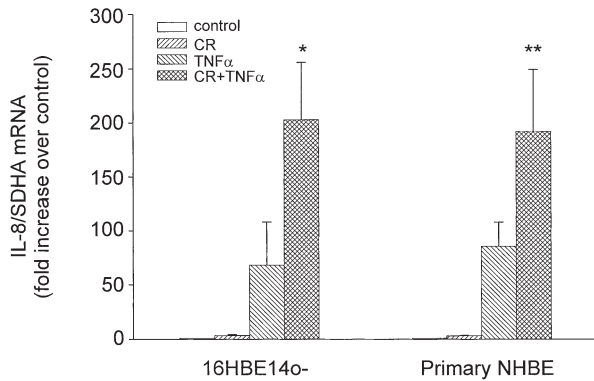


Figure 1. Cockroach extract synergistically increases TNF- α -induced IL-8 mRNA. 16HBE14o- cells or primary normal human bronchial epithelial cells were treated with cockroach extract (CR), TNF- α , or both for 4 h before isolation of RNA. Quantitative real-time PCR was performed using primers for IL-8 and SDHA, and data calculated as described in MATERIALS AND METHODS. Data represent means $\pm$ SEM for 3–5 experiments. *Significantly higher than TNF- α alone ($n = 4$, $P = 0.023$, ANOVA). **Significantly higher than TNF- α alone ($n = 4$, $P = 0.042$, ANOVA).

able to synergistically increase TNF- α -induced IL-8 promoter activity. These data suggest that although AP-1 is an important site for optimal promoter activation, it is not required for cockroach extract-induced synergy.

To confirm these data, we investigated the role of cockroach extract and TNF- α on AP-1 translocation to the nucleus and DNA binding. We transiently transfected 16HBE14o- cells with a cDNA encoding AP-1-responsive promoter elements subcloned into luciferase (AP-1 Luc). Treatment of cells with TNF- α or cockroach extract alone slightly increased AP-1 transactivation; however, there was no statistically significant increase when cells were treated with both stimuli together (Figure 2B). In addition, EMSA confirmed the promoter data (not shown). Together, these data suggest that while cockroach extract and TNF- α regulate AP-1 translocation and DNA binding, the AP-1 site is not required for synergistic effect of cockroach extract on IL-8 transcription.

Regulation of NF-IL6 by TNF- α and Cockroach Exposure

We next tested the requirement of the NF-IL6 transcription factor binding site on the synergistic increase in IL-8 by transfecting cells with the wild-type IL-8 promoter or an IL-8 construct with a mutation of the NF-IL6 binding site. There was a significant attenuation of TNF- α -induced transcription from the IL-8 promoter (Figure 3A). In addition, mutation of the NF-IL6 site totally abolished cockroach extract-induced synergy. These data demonstrate that the NF-IL6 site is an important regulator IL-8 and suggest the role by which cockroach elicits its transcriptional effect.

Next we transiently transfected cells with MFG-82, a luciferase reporter gene fused to NF-IL6 (c/EBP β)-dependent myelomonocytic growth factor (MGF), which contains an additional region harboring two NF-IL6 binding sites (21). Treatment of cells with cockroach extract or TNF- α alone increased MGF-82 activation. In addition, a further increase in MGF-82 was detected when cockroach extract and TNF- α were added together (Figure 3B). These data were confirmed using the EMSA. Treatment of cells with either cockroach extract or TNF- α alone resulted in NF-IL6 translocation to the nucleus (Figure 3C). However, a noteworthy increase in NF-IL6-DNA binding was

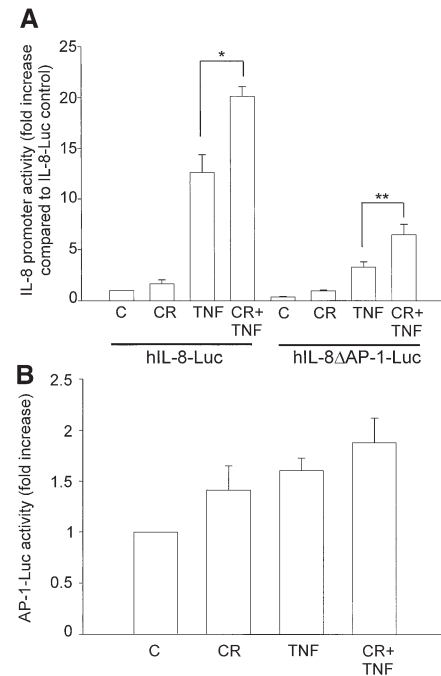


Figure 2. Cockroach extract synergistically increases IL-8 promoter activity independent of AP-1. (A) 16HBE14o- human bronchial epithelial cells were transiently transfected with β -galactosidase and either the wild-type hIL-8-Luc or hIL-8 Δ AP-1-Luc. Selected cultures were treated with cockroach extract and/or TNF- α for 16 h. Control (C), cockroach extract (CR), TNF- α (TNF), CR+TNF- α (CR+TNF). Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control vector for the IL-8-Luc plasmid ($n = 5$; * $P < 0.001$; ** $P < 0.001$, ANOVA). (B) Cells were transiently transfected with a cDNA encoding AP-1 responsive promoter elements subcloned into luciferase (AP-1 Luc). Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control vector for the IL-8-Luc plasmid ($n = 5$, $P = 0.061$; ANOVA).

detected when cells were treated simultaneously with cockroach extract and TNF- α . Next, we used the TransAM colorimetric transcription factor kit specific for NF-IL6 (c/EBP β) to quantitatively determine NF-IL6-DNA binding. Treatment of cells with cockroach extract or TNF- α alone increased NF-IL6-DNA binding. In addition, a significant increase in NF-IL6-DNA binding occurred when cells were treated with cockroach extract and TNF- α together (Figure 3D). Lastly, to further confirm that NF-IL6 and not another of the c/EBP family members is responsible for this effect, we transiently transfected cells with a dominant-negative NF-IL6 expression vector (LIP). Expression of the dominant-negative NF-IL6 vector abolished the synergistic increase in IL-8 transcription after treatment with cockroach extract and TNF- α (Figure 3E). Taken together, these data illustrate (1) the importance of the NF-IL6 site in cockroach extract-induced synergy, and (2) that although both cockroach extract and TNF- α increase NF-IL6 activation, it is the synergistic increase in NF-IL6 that is responsible for increased transcription from the IL-8 promoter.

Cockroach Proteases and PAR-2 Are Responsible for Translocation of NF-IL6

We have previously shown that cockroach extract contains serine protease activity, which is responsible for ERK activation (3, 5). To test for the involvement of cockroach proteases in NF-IL6 translocation and DNA binding, we abolished active serine

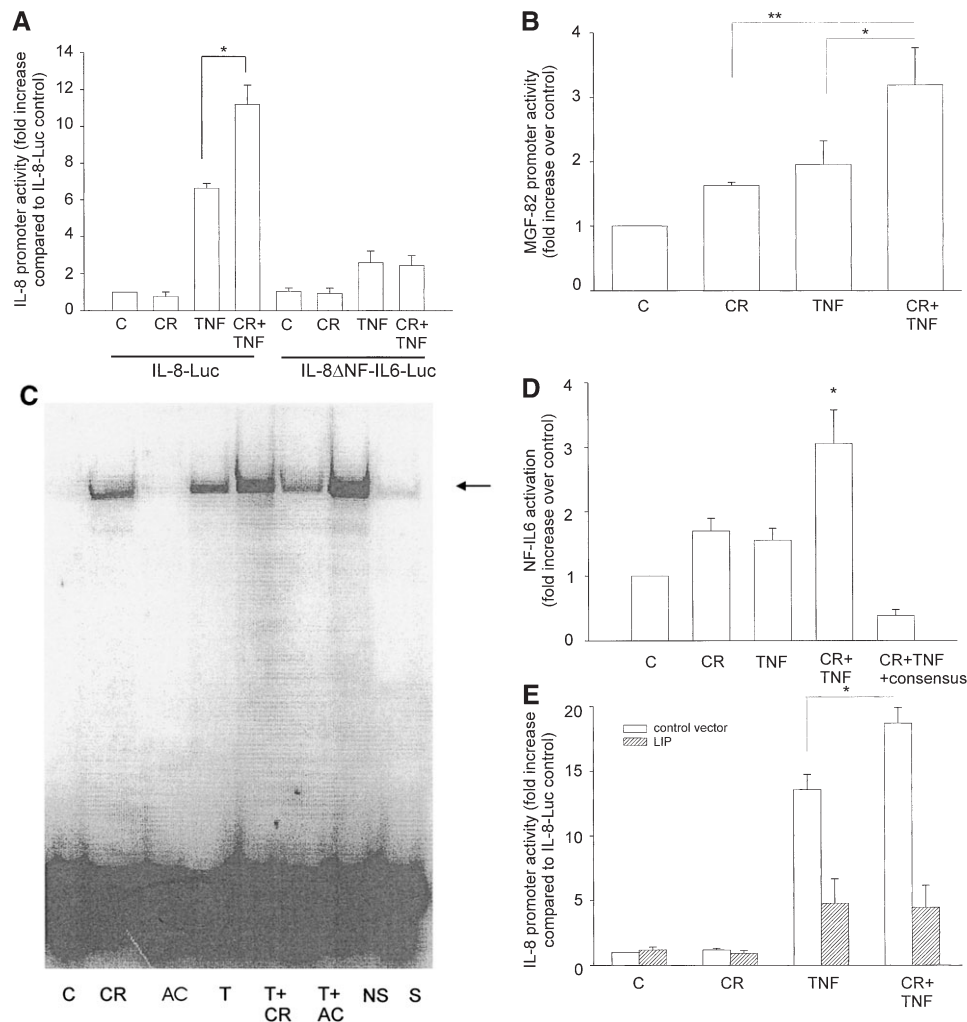


Figure 3. Cockroach extract requires and activates the NF-IL6 site in the IL-8 promoter. (A) Cells were transiently transfected with β -galactosidase and either the hIL-8-Luc or hIL-8 Δ NF-IL6-Luc. Selected cultures were treated with cockroach extract and/or TNF- α for 16 h. Abbreviations are as described in the legend to Figure 1. Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control vector for the IL-8-Luc plasmid ($n = 4$; $*P < 0.001$, ANOVA). (B) Cells were transiently transfected with a cDNA encoding NF-IL6 responsive promoter elements subcloned into luciferase (MGF-82 Luc). Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control ($n = 6$, $*P < 0.018$ compared with TNF- α alone; $**P < 0.011$ compared with CR alone; ANOVA). (C) Cells were treated with cockroach extract (CR), cockroach extract pretreated with aprotinin (AC), and/or TNF- α . Nuclear extracts were obtained, incubated with a ^{32} P end-labeled double-stranded NF-IL6 oligonucleotide, and resolved on a gel. This experiment was repeated twice. (D) Nuclear extracts were obtained and activated NF-IL6 was detected by binding a consensus oligonucleotide. A primary antibody for c/EBP β (NF-IL6) detected specific NF-IL6-DNA binding by ELISA. Wild-type consensus sequence for NF-IL6 determined specificity for NF-IL6. Data are expressed as fold increase compared with control ($n = 7$; $*P < 0.01$; ANOVA). (E) Cells were transiently transfected with a cDNA encoding a dominant-negative NF-IL6 (LIP) or vector control and IL-8 Luc. Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control vector for the IL-8-Luc plasmid ($n = 4$; $*P < 0.003$, ANOVA).

protease activity using aprotinin. Inhibition of protease activity abolished cockroach extract-induced synergistic increases in NF-IL6 translocation and DNA binding were abolished in both 16HBE14o- cells (Figure 3C) and primary human bronchial epithelium (Figure 4). Cockroach serine proteases have been previously shown to activate PAR-2, but not PAR-1, in human bronchial epithelial cells (5). To test whether PAR-2 activates NF-IL6, cells were treated with the selective PAR-2 agonist SLIGKV or the selective PAR-1 agonist TFRIFD. As shown in Figure 5 (NF-IL6 promoter assay) and confirmed by NF-IL6 ELISA (not shown), selective activation of PAR-2, but not PAR-1, results in NF-IL6 translocation and DNA binding. Together these studies suggest the importance of the cockroach

proteases and PAR-2 in regulating NF-IL6 translocation and DNA binding to transcriptionally control IL-8 expression.

Cockroach Extract and ERK Regulate NF-IL6-DNA Binding

We have previously shown that cockroach extract induces an intracellular signaling cascade involving the G-coupled protein receptor PAR-2, Ras, MEK, and ERK (5). To confirm that cockroach extract-induced translocation and DNA binding were mediated by ERK activation, we transiently transfected 16HBE14o- cells with the MGF-82-Luc plasmid and then pretreated with PD98059, a specific MEK inhibitor, before treatment. Inhibition of ERK totally abolished cockroach-induced MGF-82-Luc activation and abolished the synergy (data not

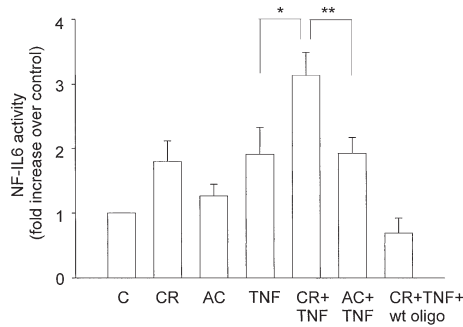


Figure 4. Cockroach proteases mediate NF-IL6-DNA binding. Primary normal human bronchial epithelial cells were treated with cockroach extract (CR), aprotinin-pretreated CR (AC), or TNF- α . Excess wild-type NF-IL6 oligonucleotide (wt oligo) was added in some cases to show specificity of the assay. Nuclear extracts were obtained and NF-IL6-DNA binding was detected by ELISA. Data are expressed as fold increase compared with control ($n = 4$; * $P < 0.02$, ** $P < 0.008$, ANOVA).

shown). Similar data were found when we assayed for NF-IL6-DNA binding using the transcription factor assay ELISA (Figure 6). Together, these data suggest that the increase in NF-IL6 by either cockroach extract or TNF- α is due to the activation of ERK.

Cockroach Proteases Do Not Regulate NF- κ B

To rule out any possible cross-talk between the cockroach extract signaling pathway and further activation of NF- κ B, we asked whether cockroach extract could regulate NF- κ B translocation in the absence or presence of TNF- α . Cockroach extract had no effect on TNF- α -induced NF- κ B translocation as determined by promoter assay (Figure 7) or electrophoretic gel shift assay (data not shown). These data demonstrate that the signaling pathway regulated by cockroach extract does not activate NF- κ B.

DISCUSSION

In this study we determined the transcriptional mechanism by which German cockroach extract elicits a synergistic increase in TNF- α -mediated IL-8 expression. Because cockroach extract activates ERK (5), we focused on the transcription factors AP-1

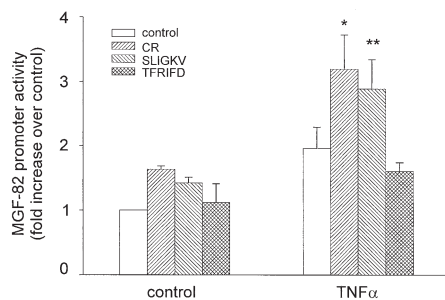


Figure 5. Selective activation of PAR-2 increases NF-IL6 translocation. Cells were transiently transfected with a cDNA encoding NF-IL6 responsive promoter elements subcloned into luciferase (MGF-82 Luc). Cells were treated with the selective PAR-2-activating peptide SLIGKV or the selective PAR-1-activating peptide TFRIFD. Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control ($n = 4$ * $P < 0.008$, ** $P < 0.022$ over TNF- α alone; ANOVA).

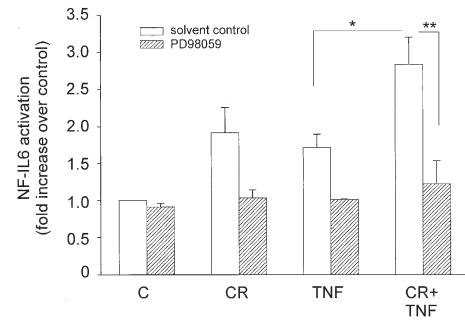


Figure 6. ERK is required for cockroach extract-induced NF-IL6-DNA binding. 16HBE14o- cells were pretreated with the MEK inhibitor PD98059 for 1 h before the addition of cockroach extract (CR), TNF- α , or both. Nuclear extracts were obtained and NF-IL6-DNA binding was detected by ELISA. Data are expressed as fold increase compared with control ($n = 3$; * $P < 0.022$, ** $P < 0.004$, ANOVA).

and NF-IL6. Our data confirmed that AP-1 is a basal level enhancer of IL-8 transcription in human airway epithelial cells (16). TNF- α induced AP-1 translocation and DNA binding, but the AP-1 site is not required for TNF- α -mediated transcription from the IL-8 promoter because a similar fold-increase in IL-8 promoter activity was found with either the wild-type or AP-1 mutant IL-8 promoter construct. In addition, mutation of the AP-1 binding site in the context of the wild-type IL-8 promoter had no effect on cockroach extract-induced synergy. This suggests that AP-1 is not the prime regulatory site on the IL-8 promoter and is not responsible for cockroach extract-induced synergy of IL-8 transcription.

Our data also show that TNF- α induced NF-IL6 translocation and DNA binding. In addition, TNF- α -induced promoter activation was inhibited when the NF-IL6 binding site was mutated in the context of the wild-type promoter, suggesting that NF-IL6 plays a role in TNF- α -induced transcription from the IL-8 promoter. These data are consistent with those of Vlahopoulos and coworkers, who showed that a mutation in the NF-IL6 binding site of the IL-8 promoter partially inhibited TNF- α -induced IL-8 transcription in a human histiocytoma lymphoma cell line (23). These data suggest that although TNF- α induces NF-IL6 translocation and DNA binding, the NF-IL6 site is not the prime regulatory site for TNF- α -induced transcription from the IL-8 promoter.

Importantly, mutation of the NF-IL6 site in the IL-8 promoter

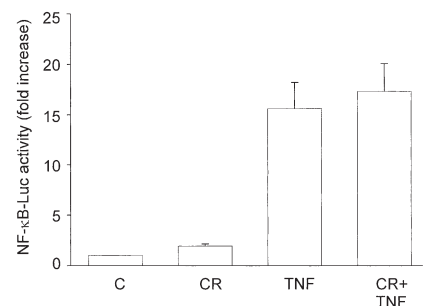


Figure 7. Cells were transiently transfected with a cDNA encoding NF- κ B responsive promoter elements subcloned into luciferase (NF- κ B Luc). Data are expressed as the fold increase in luciferase units/ β -galactosidase/h relative to the control vector for the IL-8-Luc plasmid ($n = 10$, $P = 0.0524$; ANOVA).

abolished cockroach extract-induced synergy, suggesting its crucial role in cockroach extract-induced regulation of IL-8. Cockroach extract alone mediated NF-IL6 translocation and DNA binding as determined by gel shift analysis, ELISA, and luciferase promoter studies. Treatment of cells with cockroach extract and TNF- α together further increased NF-IL6 translocation and DNA binding. These data confirm that cockroach extract synergistically increased IL-8 transcription by increasing NF-IL6 translocation and DNA binding. In addition, by inhibiting either the serine protease in cockroach extract or ERK, we abolish cockroach extract-induced activation of NF-IL6. Selective activation of PAR-2, but not PAR-1, also regulates NF-IL6 activity, suggesting that the PAR-2 activation of ERK that we previously characterized (5) is mediating this effect. Although we were unable to supershift the NF-IL6 band with the few existing antibodies commercially available, we are confident that it is indeed NF-IL6 and not one of the other c/EBP family members mediating this effect, because (1) overexpression of the dominant-negative NF-IL6 abolished cockroach extract-induced synergy and (2) the antibody used in the NF-IL6 transcription factor ELISA was specific for NF-IL6 (c/EBP β).

It is thought that NF- κ B and NF-IL6 functionally and physically interact, and the transcriptional regulation of the IL-8 promoter depends on the ratio of NF- κ B to NF-IL6. NF-IL6 has been shown to bind weakly to the IL-8 promoter, but when NF- κ B is bound to the adjacent site, NF-IL6 shows strong cooperative binding (24). Mutation of the NF- κ B site completely abolished RSV-mediated IL-8 promoter activity, whereas mutation of the NF-IL6 site only decreases promoter activity by half (25). Maximal activation of IL-8 has been shown to be regulated by the binding of both the p65 subunit of NF- κ B and NF-IL6 transcription factors (26). In this report, TNF- α induced translocation and DNA binding of both NF-IL6 and NF- κ B to the nucleus, whereas cockroach extract resulted in NF-IL6 but not NF- κ B translocation and DNA binding. This suggests that cockroach extract is regulating IL-8 transcription by increasing the amount of NF-IL6 and altering the ratio of NF-IL6 to NF- κ B to obtain maximal stimulation of IL-8 transcription. Alone, translocation of NF-IL6 does not induce IL-8 transcription (24), which explains why cockroach extract alone is insufficient to induce IL-8 promoter activation. The many proinflammatory mediators present in the bronchoalveolar lavage fluid of individuals with asthma should provide an appropriate milieu for cockroach protease to modulate the inflammatory response.

Previous studies have examined how environmental allergens modulate inflammatory signaling. For example, the house dust mite *D. pteronyssinus* allergens Der p1 and Der p9 have been shown to directly induce proinflammatory cytokines in airway epithelial cells (1). Der p1 has been shown to induce NF- κ B translocation by increasing the phosphorylation and degradation of its inhibitory protein, I κ B α , which masks the nuclear translocation signal required for NF- κ B to move to the nucleus (27). Therefore, the induction of NF- κ B translocation could explain why dust mite Der p1 protein increased cytokine expression independent of an additional stimulus, like TNF- α .

To our knowledge, this is the first report of PAR-2 inducing NF-IL6 translocation and DNA binding. A few recent studies have explored the importance of PAR-2 in airway inflammation. For example, PAR-2 knockout mice showed attenuated cellular infiltration, specifically of neutrophils and eosinophils after OVA challenge. In the same study, PAR-2-overexpressing mice were shown to have exacerbated infiltration of eosinophils into the airways. In addition, a delayed onset of inflammation, as determined by leukocyte rolling in venules, was shown in PAR-2-deficient mice (28). The importance of PAR-2 in mediating inflammation is illustrated by the fact that inhibitors of mast cell tryptase, an

activator of PAR-2, have been shown to attenuate allergen-induced airway and inflammatory responses in sheep and have been proposed as novel therapeutics for asthma (29).

In conclusion, we have shown that the mechanism by which cockroach proteases synergistically increase TNF- α -induced IL-8 expression is through the transcriptional regulation of NF-IL6 binding. Although cockroach proteases do not act alone in regulating IL-8 expression, in the presence of a stimulus, like TNF- α , exacerbations of inflammation could occur. Further studies are underway to determine the serine protease responsible for this effect. In addition, further understanding of the role of proteases in modulating the inflammatory response in airways could unveil novel therapeutic interventions for the treatment of asthma.

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