The Journal of Immunology

Activation of Eotaxin Gene Transcription by NF- κB and STAT6 in Human Airway Epithelial Cells

Satoshi Matsukura, Cristiana Stellato, James R. Plitt, Carol Bickel, Katsushi Miura, Steve N. Georas, Vincenzo Casolaro and Robert P. Schleimer

J Immunol 1999; 163:6876-6883; ;
http://www.jimmunol.org/content/163/12/6876

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Activation of Eotaxin Gene Transcription by NF-κB and STAT6 in Human Airway Epithelial Cells[1]

Satoshi Matsukura, Cristiana Stellato, James R. Plitt, Carol Bickel, Katsushi Miura, Steve N. Georas, Vincenzo Casolaro, and Robert P. Schleimer[2]

The C-C chemokine eotaxin is a potent chemoattractant for eosinophils and probably plays an important role in the pathogenesis of asthma, although the mechanisms of its regulation are not well known. Airway epithelial cells express eotaxin mRNA and protein after stimulation with a variety of cytokines. We focused on the molecular mechanisms of eotaxin gene regulation by TNF-α and IL-4 in the airway epithelial cell line, BEAS-2B. Cells were transfected with luciferase reporter plasmids, which contained up to 1363 bp of the eotaxin promoter. Eotaxin promoter activity was increased by TNF-α (2.5-fold) and IL-4 (1.5-fold), respectively. The combination of TNF-α and IL-4 produced 3.6-fold activation of the eotaxin promoter. The eotaxin promoter contains overlapping consensus binding sites for transcription factors, NF-κB and STAT6, which are known to mediate responses to TNF-α and IL-4, respectively. Electrophoretic mobility shift assays revealed NF-κB binding after TNF-α stimulation and STAT6 binding after IL-4 stimulation using a DNA probe derived from the eotaxin promoter. Mutant plasmids were generated to define the roles of these transcription factors in eotaxin promoter activity. TNF-α stimulation, but not IL-4 stimulation, was lost in plasmids mutated at the NF-κB binding site, whereas IL-4 stimulation, but not TNF-α stimulation, was lost in plasmids mutated at the STAT6 binding site. When both sites were mutated, all transcriptional activation was lost. These results imply that TNF-α and IL-4 stimulate expression of the eotaxin gene by activating NF-κB and STAT6. ***The Journal of Immunology*****, 1999, 163: 6876–6883.**

Allergic diseases, such as asthma, are characterized by the infiltration of eosinophils and lymphocytes into the sites of inflammation. Many recent studies have implicated C-C chemokines in this process. Eotaxin is a C-C chemokine originally discovered as the predominant eosinophil chemoattractant in the airways of Ag-challenged guinea pigs (1). It mediates eosinophil migration via the CCR3 receptor and also has chemotactic activity for basophils and T cells, especially Th2 cells (2, 3). An important role for eotaxin in allergic disease has been suggested based on studies in animal models (1, 4, 5) and humans (6, 7) in vivo. Eotaxin is overexpressed in the airway epithelium of asthmatics. As has previously been found for RANTES (8, 9), histological studies have shown that a primary cell type expressing eotaxin is the airway epithelial cell (6, 7). Investigators have reported up-regulation of the expression of the eotaxin gene by proinflammatory cytokines, such as TNF-α and IL-1 (10). Antigen-induced up-regulation has been attributed to T cells, especially Th2 cells (11, 12). Cultured airway epithelial cells have been reported to express eotaxin (10, 13). Based upon the preceding, an analysis of the regulation of eotaxin expression in airway epithelial cells is likely to yield information relevant to the pathogenesis of allergic inflammation.

Mochizuki et al. (14) reported that TNF-α and IL-4 synergistically stimulated eotaxin expression in fibroblasts. We have found a similar synergistic effect of TNF-α and IL-4 in the induction of eotaxin mRNA and protein expression in airway epithelial cells (15). Up-regulation of eotaxin expression by TNF-α and IL-4 might be expected to occur in asthma, because TNF-α and IL-4 are both implicated in the pathogenesis of asthma. TNF-α is known to be produced by basophils, mast cells, monocytes, and macrophages (16, 17). IL-4 is a pivotal cytokine associated with allergic disease that supports Th2 development and is produced by Th2 cells, basophils, and mast cells (18–20).

The mechanisms regulating eotaxin expression in airway epithelial cells are presently unknown. Many putative binding sites for transcription factors are identified in the eotaxin promoter. Putative binding sites for NF-κB and STAT6 coexist within the proximal region of the eotaxin promoter. NF-κB is a key regulator of immune and inflammatory genes (21). NF-κB is comprised of a homodimer or heterodimer of Rel family proteins, including NF-κB1(p50/p105), NF-κB2(p52/p100), RelA(p65), RelB, and c-Rel. It is sequestered in an inactive form in the cytoplasm by the inhibitory molecules known as IκB. TNF-α activates NF-κB by inducing the phosphorylation and degradation of IκB and leads to the translocation of NF-κB to the nucleus where it can bind to specific promoter binding sites (21). Many cell types are reported to express NF-κB, including airway epithelial cells, and NF-κB has been implicated in epithelial cell responses (22, 23). IL-4 has been shown to trigger tyrosine phosphorylation of a latent cytoplasmic transcription factor termed STAT6 via receptor-associated Janus kinase-1 (JAK1)[3] and JAK3. Phosphorylated STAT6 assembles in a dimeric form, translocates into the nucleus, and *trans*-activates genes containing specific regulatory elements (24, 25).

Division of Clinical Immunology and Allergy, Johns Hopkins Asthma and Allergy Center, Baltimore, MD 21224

Received for publication May 27, 1999. Accepted for publication September 22, 1999.

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

[1] This work was supported by National Institutes of Health Grants RO1AR31891 and AI44885.

[2] Address correspondence and reprint requests to Dr. Robert P. Schleimer, Johns Hopkins Asthma and Allergy Center, Room 3A.62, 5501 Hopkins Bayview Circle, Baltimore, MD 21224-6801. E-mail address: rschleim@welch.jhu.edu

[3] Abbreviation used in this paper: JAK, Janus kinase.

0022-1767/99/$02.00

Several cell types have been shown to express STAT6; however, minimal data are available concerning airway epithelial cells (26).

In the present study we investigated the possible roles of NF-κB and STAT6 in the transcriptional regulation of the eotaxin gene in airway epithelial cells. We show that overlapping elements for both factors within the proximal eotaxin promoter mediate the transcriptional induction of reporter gene constructs by TNF-α and IL-4. We also demonstrate that TNF-α activated NF-κB and IL-4 activated STAT6 in airway epithelial cells. These findings provide insight into the mechanisms of epithelial eotaxin expression in allergic diseases.

Materials and Methods

Cell culture

BEAS-2B is a human airway epithelial cell line transformed with adenovirus 12-SV40 hybrid virus (a gift from Dr. Curtis Harris) (27). BEAS-2B cells were cultured in Hanks' F12/DMEM (Biofluids, Rockville, MD) with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 ng/ml streptomycin (Life Technologies-BRL, Gaithersburg, MD) at 37°C with 5% CO_2 in humidified air.

Amplification and cloning of the eotaxin promoter

The promoter region of eotaxin was amplified by the method of Siebert et al. using the Genome Walker kit (Clontech, Palo Alto, CA) (28). The first PCR product was amplified from adaptor-ligated genomic DNA fragments using 5′ adaptor primer and a primer derived from the eotaxin exon I sequence (5′-TAGCAGCTGCCTTCAGCCCCCAGGGG-3) (29). The primary PCR reaction mixture was diluted and used as a template for a nested PCR reaction using the nested adaptor primer and the nested primer derived from the eotaxin 5′-upstream sequence (5-ACTTCTGTGGCTGCT GCTCATAG-3). The derived PCR product was cloned using the TA cloning kit (Invitrogen, San Diego, CA), and the construct is referred to as TA-Eotax. Recombinant clones were selected based on PCR screens of derived colonies. Plasmids were isolated using the QIAGEN plasmid purification kit (Qiagen, Santa Clarita, CA). Cloned DNA sequences were determined by the dideoxy method at Johns Hopkins DNA Analysis Facility.

Construction of eotaxin promoter-luciferase reporter plasmids

A 1363-bp fragment of the promoter region of the eotaxin gene (site −1363 to −1) was amplified by PCR using a sense primer containing a restriction site for *Mlu*I and an antisense primer containing a restriction site for *Bgl*II. The amplified PCR product was cloned into a TA cloning vector and digested with *Mlu*I and *Bgl*II (New England Biolabs, Beverly, MA). The resulting fragment was cloned into the *Mlu*I and *Bgl*II sites of a promoterless luciferase reporter pGL3-Basic vector (Promega, Madison, WI), and the construct is referred to as pEotx.1363. The pEotx.478 plasmid was constructed by digesting TA-Eotx.1363 with *Sac*I and *Bgl*II and cloning the resulting 478-bp eotaxin promoter fragment into the *Sac*I and *Bgl*II sites of the pGL3-Basic vector. The pEotax.300 plasmid was constructed by 5′ end digestion of the pEotax.1363 plasmid using exonuclease III and S1 nuclease (Promega). Digested linear vector was circularized by DNA ligase after Klenow DNA polymerase treatment (Promega). The sequence of each plasmid insert DNA was determined by the dideoxy method using the fmol DNA Sequencing System (Promega).

Putative transcription factor binding sites in pEotx.1360 were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), using pEotx.1363 as a template. Constructs pEotx.M-1, -2, and -3 were synthesized by temperature cycling using site-mutated primers and PfuTurbo DNA polymerase (Stratagene). Nonmutated template plasmid was digested with *Dpn*I endonuclease (Stratagene). The mutated plasmid was transformed into XL1-Blue cells (Stratagene) and purified using the Qiagen plasmid purification kit. Mutations were selected based on the results of EMSA with mutant probes for each transcription factor (Table I). The sequence of each plasmid was determined by the method mentioned above.

Transient transfections and luciferase assays

BEAS-2B cells were seeded into six-well plates and allowed to grow to 50–70% confluence. Cells were transfected with 3 μl of Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) and 1 μg of the plasmids indicated in the text and incubated in 2 ml of medium. Cytokine was added 24 h after transfection. Six hours after cytokine treatment, cells were washed twice with Ca^{2+}- and Mg^{2+}-free HBSS (Life Technologies) and solubilized by incubation in 300 μl of reporter lysis buffer for 15 min (Promega). Cells were transferred to microtubes and centrifuged to pellet cellular debris, and the supernatant was stored at −70°C. Luciferase activity was measured using the Luciferase Assay System (Promega) and a luminometer (Analytic Luminescence Laboratories, Sparks, MD). The protein concentration of the samples was measured using the Bradford protein dye reagent (Bio-Rad, Hercules, CA), and the relative luciferase activity was normalized to the protein concentration.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared by the modified method of Schreiber et al. (30). The BEAS-2B cells were stimulated as indicated and then harvested with a scraper after washing twice with HBSS. The cells were washed with buffer A (10 mM HEPES, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10 μg/ml leupeptin; Life Technologies). The cell pellet was resuspended in buffer B (buffer A containing 0.2% Nonidet P-40) and incubated for 2 min. Nuclei were pelleted by centrifugation and resuspended in buffer C (buffer A containing 0.25 M sucrose). Nuclei were again pelleted, then resuspended in buffer D (50 mM HEPES, 400 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10 μg/ml leupeptin) and incubated with shaking for 30 min. All procedures were performed on ice. The mixture was centrifuged, and the supernatant was stored at −70°C.

Whole cell extracts were prepared by modification of a method previously reported (26, 31). Cells were harvested as described above. The cells were resuspended in lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 0.2 mg/ml leupeptin, 1 mM Na_3VO_4, and 50 mM NaF). Cells were sonicated for 10 s with an ultrasonic cell disrupter (Heat System Ultrasonics, Farmingdale, NY) and then incubated for 5 min. All procedures were performed on ice. Lysates were centrifuged, and the supernatant was collected and stored at −70°C.

DNA-protein binding assays were based on the modified methods previously reported (31, 32). Aliquots of 5 μg of nuclear extracts or whole cell extracts were incubated in 10 μl of total reaction volume containing 10 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 50 μg/ml poly(dI-dC), 0.1 mg/ml BSA, and 50 mM KCl (Life Technologies) for 5 min with or without unlabeled oligonucleotide probe. The ^{32}P-labeled oligonucleotide probe was added to the reaction mixture and then incubated for 20 min. In some experiments, Abs against p50(NF-κB1), p65(RelA), RelB, c-Rel, p52(NF-κB2), STAT1, STAT6 (Santa Cruz Biotechnology, Santa Cruz, CA), or isotype-matched antisera were incubated with the mixture for 30 min after incubation with labeled probe. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel with 0.5 × TBE buffer. The gels were dried and analyzed by autoradiography. The sequences of the oligonucleotide probes used are shown in Table I.

Immunoprecipitation and Western blot

Western blot of nuclear extracts was performed to assess translocation of STAT6 into nuclei as reported previously (33). Fifty micrograms of nuclear extracts or 2 μg of recombinant STAT6 protein (a gift from Dr. Ulrike Schindler) were incubated with 1 μg of anti-STAT6 Ab prebound to protein G-Sepharose beads (Pharmacia Biotech, Piscataway, NJ) for 2 h at 4°C with gentle rotation. After washing three times with the lysis buffer, the immunoprecipitated proteins were boiled for 5 min in the sample buffer containing 0.25 M Tris-HCl, 10% glycerol, 5% SDS, and 0.025% 2-ME (Sigma, St. Louis, MO). The samples were then subjected to 10% Tris-glycine gradient gel electrophoresis (NOVEX, San Diego, CA) and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). The membrane was blocked with 5% nonfat milk powder in TBST (50 mM Tris, 0.15 M NaCl, and 0.05% Tween-20), incubated with 1 μg/ml rabbit anti-STAT6 Ab (Santa Cruz Biotechnology) in TBST for 3 h, washed with TBST, and then incubated with anti-rabbit Ig Ab (Amersham) for 1 h. After extensive washing with TBST, chemiluminescent substrate was added (ECL Western blot detection system, Amersham), and the membrane was subjected to autoradiography.

Statistical analysis

Analyses of data from transfection/luciferase assays were performed using StatView II (Abacus Concepts, Berkeley, CA). Data are expressed as the mean ± SE. Statistical differences were determined by ANOVA with Fisher's protected least significant difference test.

Table I. *Sequences of oligonucleotides for EMSA and proximal region of eotaxin promoter-reporter plasmids*[a]

Probe/ Oligo	Sequence & putative binding sites	Plasmid
wild type	GGCTTCCCTGGAATCTCCCACA	pEotx. 1363
	STAT6 NF-κB	
m1	GGCAGCCCTGGAATCTCCCACA	pEotx. M1
m2	GGCTTCCCTGGAATCTGGGACA	pEotx. M2
m3	GGCTTCCCTCCAATCTCCCACA	pEotx. M3

[a] Putative binding sites for STAT6 and NF-κB are indicated by a box. Mutated sequences are underlined.

Results

To study the transcriptional regulation of the eotaxin gene, we cloned a region of the gene extending to bp −1363 upstream of the transcription start site using a genomic library. The sequence of this region is shown in Fig. 1. It contains a TATA box (at bp −43 to −39) and consensus binding sites for NF-κB (at bp −68 to −59), STAT6 (at bp −74 to −65), AP-1 (at bp −1082 to −1076), and C/EBP (at bp −1278 to −1270). The overall sequence is similar, but not identical, to those published by Garcia-Zepeda et al. and Hein et al. (34, 35). This fragment was cloned into a luciferase reporter plasmid for further functional analysis.

TNF-α (0.1, 1, 10, and 100 ng/ml) dose dependently increased luciferase activity in BEAS-2B cells transfected with the pEotx.1363 plasmid (Fig. 2*A*). Maximum induction was observed using 100 ng/ml of TNF-α (2.5 ± 0.1-fold; $p < 0.05$ compared with control). IL-4 (0.5, 5, and 50 ng/ml) also increased luciferase activity dose dependently, although modestly compared with TNF-α (Fig. 2*B*). Maximum induction was observed using 50 ng/ml of IL-4 (1.5 ± 0.04-fold; $p < 0.05$ compared with control). The combination of 100 ng/ml TNF-α and 50 ng/ml IL-4 additively activated the eotaxin promoter (3.6 ± 0.2-fold; $p < 0.05$ compared with control; Fig. 3*A*). Similar results were obtained using cells transfected with reporter plasmids containing either a 478-bp fragment or a 300-bp fragment of the eotaxin promoter (Fig. 3, *B* and *C*).

Many effects of TNF-α are known to be mediated via activation of NF-κB, while IL-4 is known to activate STAT6 via JAK1 and JAK3. We therefore used EMSA to determine whether stimulation with these cytokines results in formation of active NF-κB or STAT6. EMSA with nuclear extracts from BEAS-2B cells stimulated with 10 ng/ml TNF-α resulted in the formation of a strong binding complex (complex 1) using a DNA probe spanning the putative binding sites for STAT6 and NF-κB in the eotaxin promoter (Fig. 4*A*). This binding complex was blocked or supershifted by Abs to p50 and p65 NF-κB Rel family members. Abs against other Rel family members (RelB, c-Rel, and p52) and STAT6 did

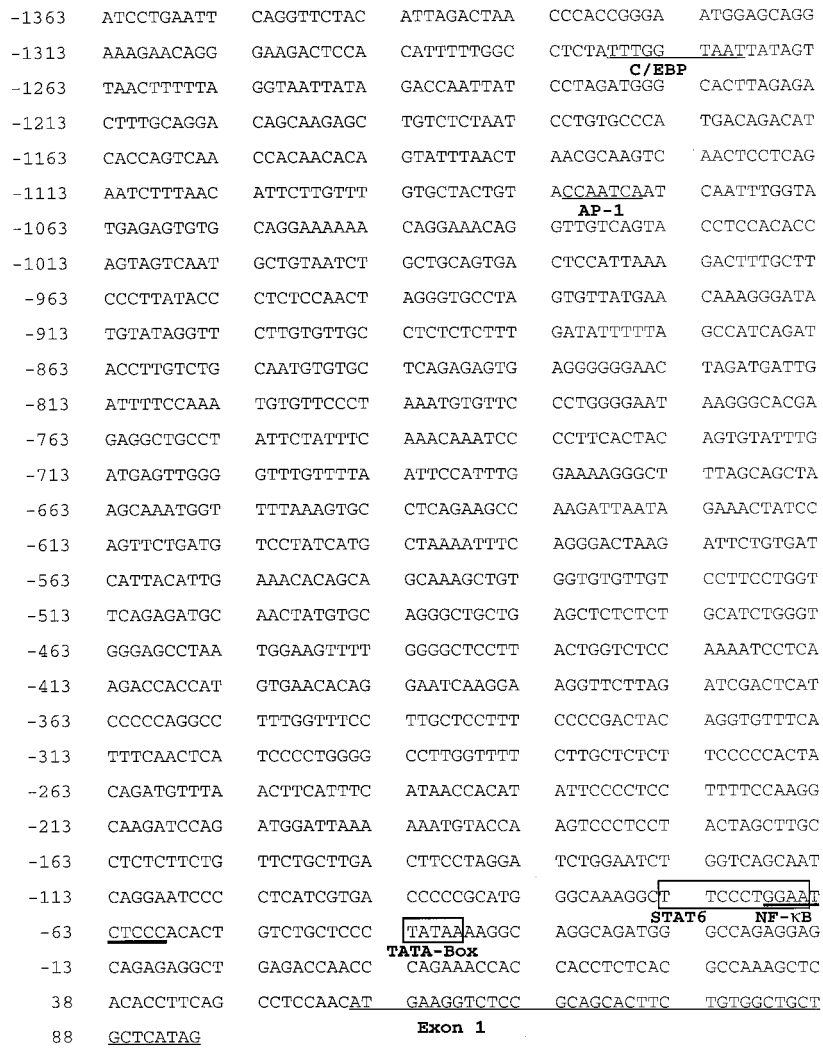

FIGURE 1. DNA sequence of the eotaxin promoter extending through bp −1363 upstream of the transcription start site. The putative binding sites for transcription factors, possibly responsible to TNF-α or IL-4, are indicated by underlines or a box. The TATA box and part of exon 1 are also indicated by underlines or a box.

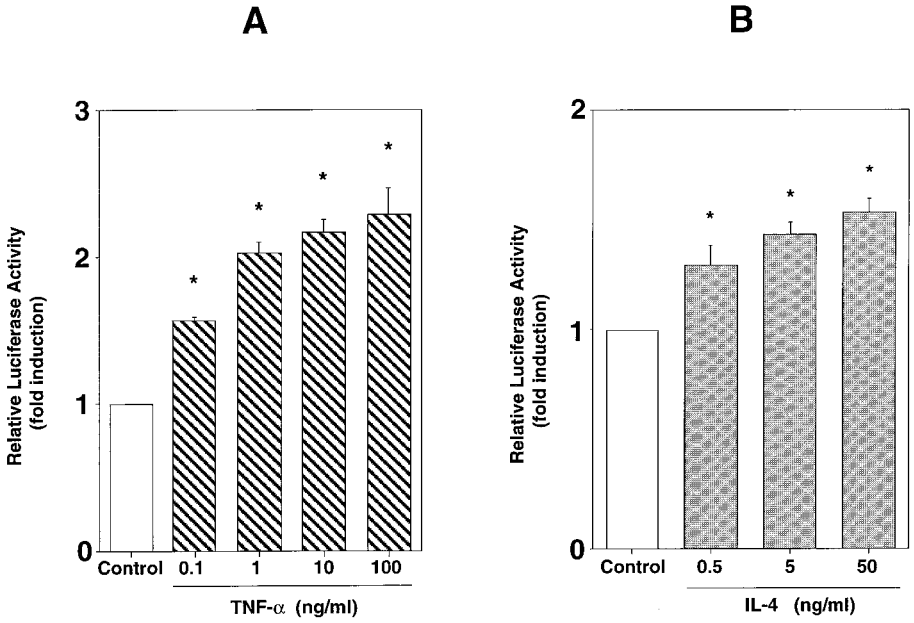

FIGURE 2. Activation of the eotaxin promoter by TNF-α and IL-4. BEAS-2B cells were transfected with a wild-type eotaxin promoter-luciferase reporter plasmid (pEotx.1363). Twenty-four hours after transfection, cells were incubated with or without the indicated concentrations of TNF-α (*A*) or IL-4 (*B*) for 6 h and then harvested. The luciferase activity was normalized to protein concentration and calculated as fold induction compared with control. The data are presented as the mean $\pm$ SE from a total of four independent experiments. $*$, $p < 0.05$ compared with control.

not affect formation of the binding complex. Preimmune rabbit and goat IgG did not influence complex formation (data not shown). The binding specificity of the NF-κB complex was further assessed by competition with unlabeled oligonucleotides. Unlabeled wild-type oligonucleotide and an oligonucleotide only mutated within the putative STAT6 binding site (referred to as m1, Table I) competed for DNA-protein complex formation (Fig. 4*B*), while two oligonucleotides carrying a mutation within the putative NF-κB binding site (referred to as m2 and m3) were ineffective. Taken together, these results suggest that TNF-α induces the formation of a sequence-specific NF-κB complex composed of p65/p50 heterodimers. IL-4 did not induce NF-κB and the combination of IL-4 and TNF-α did not alter the binding complex compared with TNF-α alone (data not shown). An unknown complex (complex 2) was formed equally in unstimulated cells or cells stimulated with TNF-α (Fig. 4, *A* and *B*). Although this constitutive complex was not inhibited by any of the Ab mentioned above (Fig. 4*A*), the complex formation was inhibited by excess of wild-type oligonucleotide and m1 (Fig. 4*B*).

The STAT6-DNA binding complex was barely detectable using nuclear extract (data not shown), presumably due to the lower efficiency of EMSA from nuclei compared with NF-κB. Therefore,

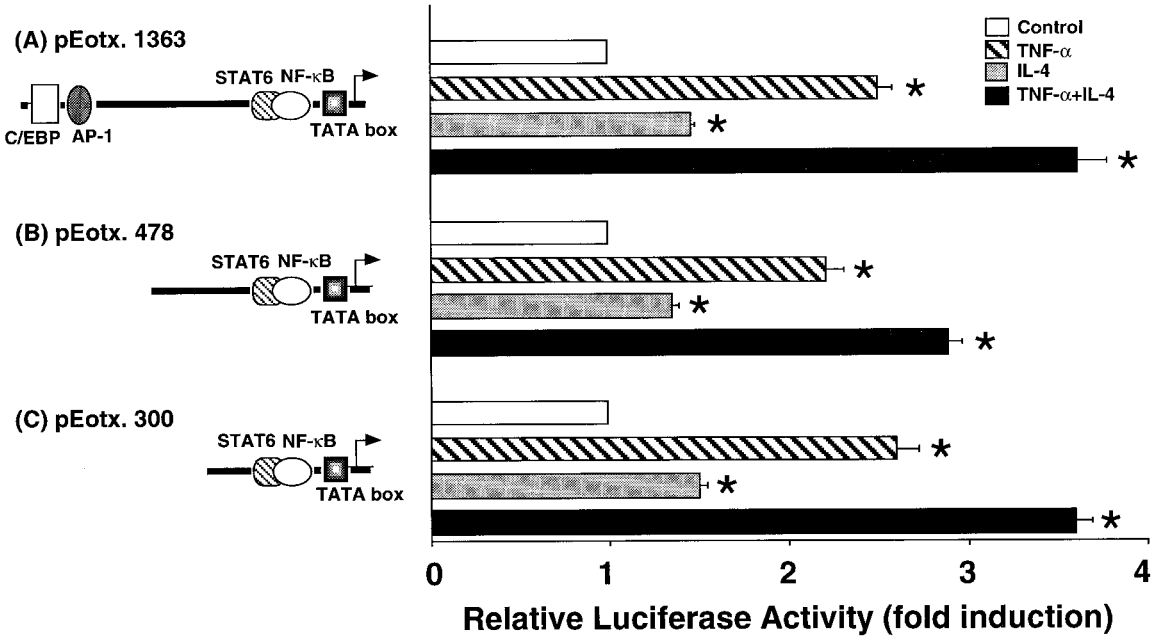

FIGURE 3. Cooperative activation of the eotaxin promoter by TNF-α and IL-4. BEAS-2B cells were transfected with the indicated eotaxin promoter-luciferase reporter plasmids, which contained 1363 bp of the proximal promoter sequence (referred to as pEotx.1363; *A*), 478 bp of the promoter (referred to as pEotx.478; *B*), or 300 bp of the promoter (referred to as pEotx.300; *C*). Twenty-four hours after transfection, cells were incubated with or without TNF-α (100 ng/ml) and/or IL-4 (50 ng/ml) for 6 h and then harvested. The luciferase activity was normalized to protein concentration and calculated as fold induction compared with the control value. The data are presented as the mean $\pm$ SE of a total of six independent experiments. $*$, $p < 0.05$ compared with control.

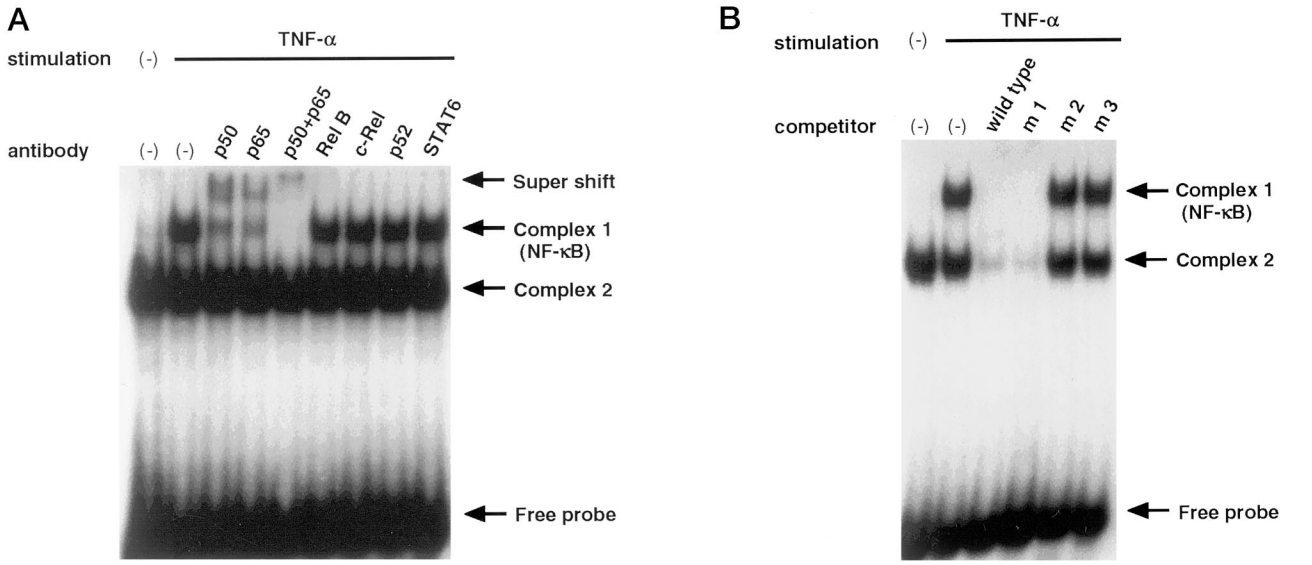

FIGURE 4. NF-κB1(p50) and RelA(p65) bind to the proximal region of the eotaxin promoter. Nuclear protein was extracted from BEAS-2B cells treated with or without TNF-α (10 ng/ml), incubated with or without the indicated Ab (*A*) or a 50-fold molar excess of unlabeled oligonucleotide (*B*) and analyzed for binding activity by EMSA using a radiolabeled oligonucleotide probe containing the eotaxin promoter sequence indicated in Table I. Results are representative of five independent experiments (*A*) or of three independent experiments (*B*).

we confirmed the ability of DNA-STAT6 binding by following experiments based on the method described by Guo et al. (26), because activated STAT6 in cytoplasm can be bound to DNA probe. EMSA with whole cell extracts from BEAS-2B cells stimulated with 10 ng/ml IL-4 were performed. A binding complex (complex 1) was induced by IL-4 and detectable in whole cell extracts using a wild-type DNA probe (Fig. 5). This binding complex was confirmed to contain STAT6 by inhibition using anti-STAT6 Ab. Abs against Rel family members and STAT1 did not influence formation of the binding complex (Fig. 5), nor did rabbit IgG (data not shown). Unlabeled wild-type oligonucleotide and the oligonucleotide mutated at only the NF-κB binding site (m2) inhibited DNA-protein binding, while oligonucleotides mutated at the STAT6 binding site (m1 and m3) were inactive. TNF-α did not induce STAT6, and the combination of IL-4 and TNF-α did not alter the STAT6-DNA binding complex compared with IL-4 alone (data not shown). Unknown complexes 2, 3, and 4 seem to be comprised of constitutively expressed proteins that bound to the eotaxin promoter. Taken together, these results indicate that IL-4 induces activation of functional STAT6 in airway epithelial cells.

Western blot of nuclear extracts, immunoprecipitated with anti-STAT6 Ab, was used to assess the translocation of STAT6 into the

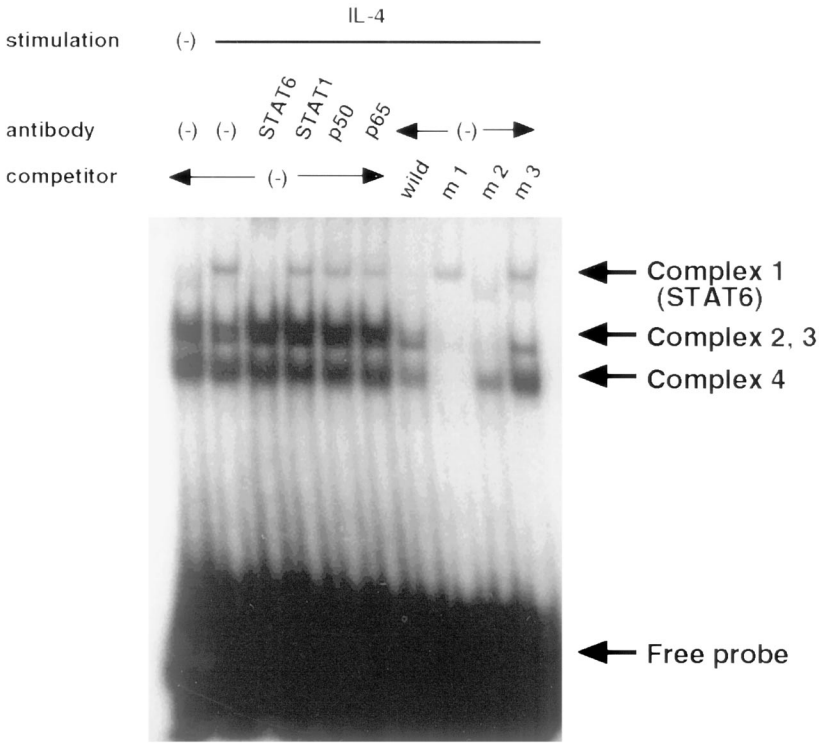

FIGURE 5. STAT6 binds to the proximal region of the eotaxin promoter. A whole cell extract was prepared from BEAS-2B cells treated with or without IL-4 (10 ng/ml). The extract was incubated with or without the indicated Ab or a 50-fold molar excess of unlabeled oligonucleotide probe and analyzed for binding activity by EMSA using a radiolabeled oligonucleotide probe derived from the eotaxin promoter indicated in Table I. Results are representative of three independent experiments.

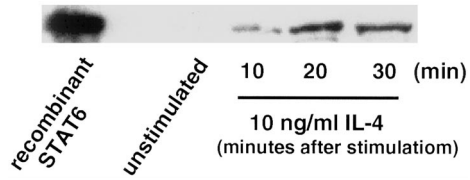

FIGURE 6. Translocation of STAT6 into the nucleus after stimulation with IL-4. Nuclear protein was extracted from BEAS-2B cells treated with or without IL-4 (10 ng/ml) for the indicated periods of time, immunoprecipitated with anti-STAT6 Ab, and analyzed by Western blot using anti-STAT6 Ab. Results are representative of two independent experiments.

nucleus following stimulation of 10 ng/ml IL-4. Recombinant STAT6 protein was used as positive control. A minimal level of STAT6 was detected from the nuclei of unstimulated BEAS-2B cells (Fig. 6). The amount of STAT6 in the nucleus increased after stimulation with IL-4.

To test the roles of NF-κB and STAT6 binding sites in transcription of the eotaxin gene, luciferase reporter plasmids with mutated proximal promoters were used. The sequences of sites in the mutant plasmids are shown in Table I. A reporter using the eotaxin promoter mutated at the binding site for STAT6 (referred to as pEotx.M1) showed total loss of IL-4 stimulation (Fig. 7*B*) and an additive effect with TNF-α, but still responded to stimulation with TNF-α. A reporter plasmid, pEotx.M2, which was mutated at the binding site of NF-κB, showed total loss of TNF-α stimulation and additive activation with IL-4 (Fig. 7*C*), but still responded to stimulation with IL-4. Neither TNF-α nor IL-4 activated the reporter, pEotx.M3, which was mutated at the overlapping binding sites for STAT6 and NF-κB (Fig. 7*D*).

Discussion

Several recent studies indicate that airway epithelial cells are a rich source of eotaxin in humans in individuals with allergic airways disease and/or after experimental allergen challenge in animals (1, 4–7). To study the regulation of expression of the eotaxin gene in epithelial cells, we isolated 1363 bp of the proximal promoter. The sequence of the derived eotaxin 5′ upstream region is compatible to the sequences that have recently been reported by two independent groups of investigators (34, 35). Luciferase reporter constructs containing this promoter region were activated by both TNF-α and IL-4. Combining these two cytokines caused an additive effect. Alone, TNF-α was a stronger stimulus than IL-4. Because a strong synergy between these two stimuli is observed at the protein level, and an intermediate synergy is observed in Northern blot studies, we suspect that post-transcriptional and/or translational mechanisms may be involved (15). Based upon the presence of a single site containing overlapping consensus response elements for both NF-κB and STAT6, we used EMSA to probe for activation of these two transcription factors. NF-κB has been known to regulate expression of several inflammatory proteins, including RANTES, IL-8, monocyte chemoattractant protein-1, inducible nitric oxide synthase, VCAM-1, and ICAM-1 (23, 36–40). STAT6 has been shown to be essential for most responses to IL-4. It activates transcription of several IL-4-induced genes, such as CD23 (the low affinity IgE receptor), MHC class II, and IgE germline and mediates Th2 development (24, 41–43).

TNF-α induced the activation of NF-κB, which bound to a DNA probe derived from the eotaxin proximal promoter in airway epithelial cells. The sequence of this site is slightly different from a consensus sequence of the NF-κB binding site (the consensus sequence is GGGRNNYYCC; the eotaxin promoter sequence is GGAATCTCCC; positions bp −68 to −59; N indicates any of nucleotides, R indicates A or G, and Y indicates C or T; see Table I) (21, 44). The proteins bound to the DNA probe were confirmed to be p50 and p65 of the NF-κB family, however, using specific Abs.

Activation of STAT6 by IL-4 was detected by EMSA using a DNA probe derived from the eotaxin promoter that contains a

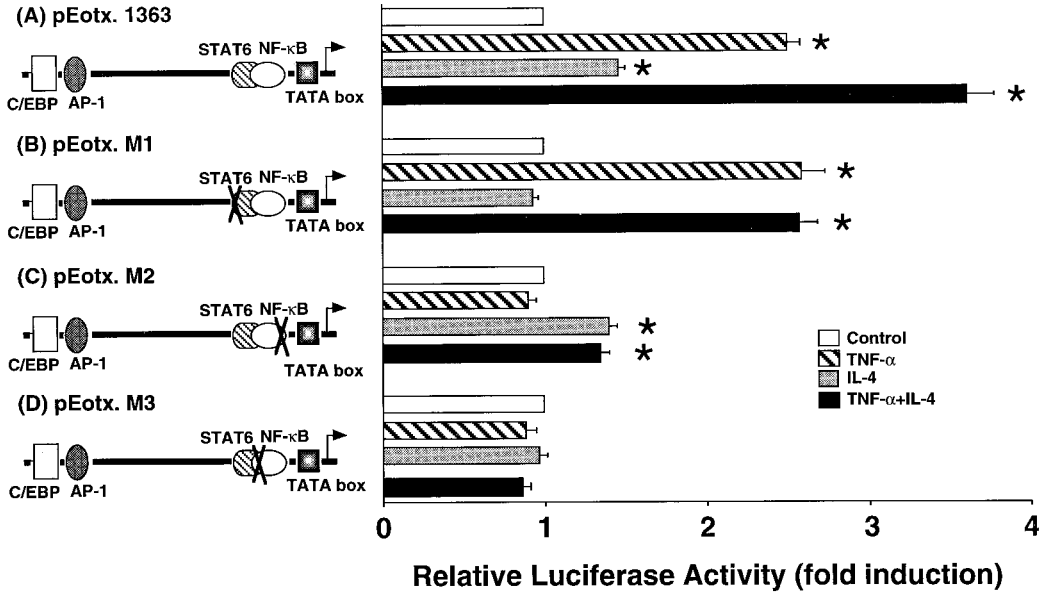

FIGURE 7. Activation of the eotaxin promoter by NF-κB and STAT6. BEAS-2B cells were transfected with either a wild-type eotaxin promoter-luciferase reporter plasmid (pEotx.1363; *A*) or a plasmid mutated at binding site for STAT6 (pEotx.M1; *B*), a plasmid mutated at binding site for NF-κB (pEotx.M2; *C*), or a plasmid mutated at the overlapping binding site for both STAT6 and NF-κB (*D*; see Table I). Twenty-four hours after transfection, cells were incubated with or without TNF-α (100 ng/ml) and/or IL-4 (50 ng/ml) for 6 h and then harvested. The luciferase activity was normalized to protein concentration and calculated as the fold induction compared with the control value. The data are presented as the mean ± SE of a total of six independent experiments. ∗, $p < 0.05$ compared with control.

putative STAT6 binding site (the consensus sequence is TTCN3/4 GAA; the eotaxin sequence is TTCCCTGGAA; positions bp −74 to −65; see Table I) (45) and also overlaps with the 5′-end of the putative NF-κB binding site mentioned above. The specificity of TNF-α-induced NF-κB and IL-4-induced STAT6 was further established by the ability of unlabeled consensus probes to inhibit respective complex formation as well as by the use of unlabeled eotaxin probe mutated at either site. Thus, we have identified a unique site in the eotaxin promoter that binds to both NF-κB and STAT6. Other investigators have reported that TNF-α can induce or enhance STAT6 activation, and IL-4 can induce activation of NF-κB (46–48); however, our results suggest that TNF-α activated NF-κB and not STAT6, while IL-4 activated STAT6 and not NF-κB. Both these transcription factors independently bound to the eotaxin promoter element in our studies.

Having established that TNF-α and IL-4 can activate eotaxin promoter reporter plasmids, and that TNF-α activated NF-κB and IL-4 activated STAT6, we next tested whether NF-κB and STAT6 were responsible for activating the reporter genes. Promoter assays using mutant plasmids showed that the putative NF-κB site at −68 to −59 is necessary for TNF-α activation of the eotaxin promoter. Likewise, results with the pEotx.M1 mutant indicate that the putative STAT6 binding site was necessary for IL-4 activation of the promoter. In both cases, the additive effects were lost when plasmids mutated at either site were used. These findings add support to the data from EMSA, suggesting that TNF-α and IL-4 activate the eotaxin promoter via NF-κB and STAT6, respectively.

TNF-α has been shown to activate NF-IL-6 (C/EBP family) and AP-1 (49, 50). Our eotaxin reporter plasmid contains consensus binding sites for both these factors as has been indicated by other investigators (34, 35). We did not detect any clear complexes from EMSA using DNA probes derived from these sequences after TNF-α treatment, however (data not shown). Although IL-4 has also been reported to activate the C/EBP family (51), no clear DNA-protein binding was observed in EMSA with a probe derived from the C/EBP consensus element contained in the eotaxin promoter (data not shown). Reporter gene assays using further truncated forms of the promoter (pEotx.478 and pEotx.300) recapitulated the findings with the 1363-bp promoter fragment. Because activation by TNF-α and IL-4 was preserved in the transfections with these plasmids that do not contain the putative binding sites for C/EBP or AP-1, these transcription factors are not likely to be essential for activation of the eotaxin promoter fragments used by TNF-α and IL-4.

The results of this study suggest that NF-κB and STAT6 act positively at a site on the eotaxin promoter containing overlapping binding sites. This contrasts with the results of Bennet et al., who reported suppression of TNF-stimulated E-selectin expression by STAT6 antagonism of NF-κB (52). They suggested that overlapping binding sites of NF-κB and STAT6 in the E-selectin promoter resulted in competition between STAT6 and NF-κB for binding. On the E-selectin promoter, there are two adjacent, but nonoverlapping, binding sites for NF-κB that are straddled by a STAT6 binding site that shares 5 bp with each NF-κB site. In the eotaxin promoter, STAT6 and NF-κB share only 4 bp for both factors; this structure may make it possible for factors to bind simultaneously to the overlapping site on the eotaxin promoter. Shen et al. and Iciek et al. have reported that NF-κB and STAT6 synergistically activate the mouse germline Cε promoter (48, 53). A similar mechanism has been reported in the human IgE germline promoter (54). In these cases, a STAT6 binding site was adjacent to a NF-κB binding site, although these sites were separated by several nucleotides. There are other reports that support an cooperation between NF-κB and STAT6 on overlapping binding sites. Isalan et al. demonstrated that zinc finger-containing proteins can generally recognize overlapping DNA binding domains based upon analysis of the crystal structure of these binding proteins (55). Although the three-dimensional crystal structures of STATs and NF-κB have been analyzed (44, 56), there are few data concerning such interactions of these two transcription factor families. Wotton et al. reported cooperative binding of Ets-1 and core binding factor to the individual sites, which are directly adjacent on the TCR β gene enhancer region of the promoter (57). Our mutagenesis experiments clearly establish independent roles for NF-κB and STAT6 in regulating the eotaxin promoter despite the intimate association of binding sites for these two factors. These reports and our data suggest that the differences in DNA motif and structure of promoter could result in different patterns of protein binding to DNA and promoter activity. Further study will be necessary to better understand the mechanism of interactions of NF-κB and STAT6 on the eotaxin promoter, especially in mediation of the synergy between TNF-α and IL-4.

Both NF-κB and STAT6 have been implicated in allergic inflammation. Yang et al. have shown that mice deficient in the p50 subunit of NF-κB protein do not mount eosinophilic lung inflammation and that eotaxin expression was inhibited compared with that in wild-type mice (58). Their data are in agreement with our findings indicating an important role for NF-κB in the regulation of eotaxin in airway epithelium. Airway eosinophilia and hypersensitivity were severely reduced in STAT6-deficient mice (59, 60). This could result from impaired Th2 development or IgE synthesis, which are mainly regulated by IL-4/STAT6. Our studies suggest that impaired eotaxin synthesis in STAT6-deficient mice may be expected to contribute to reduced eosinophil infiltration.

Acknowledgments

We thank Dr. Ulrike Schindler, Bruce S. Bochner, Lisa A. Beck, David Proud, Jean Kim, Mio Kawaguchi, and Shau-Ku Huang for excellent assistance and helpful discussions, and John E. Cumberland, John W. Schmidt, Eva Ehrlich-Kautzky, Eri Matsukura, and Bonnie Hebden for skillful assistance.

References

1. Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammations. *J. Exp. Med. 179:881.*
2. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of eotaxin receptor CCR3 by human T helper 2 cells. *Science 277:2005.*
3. Yamada, H., K. Hirai, M. Miyamasu, M. Iikura, Y. Misaki, S. Shoji, T. Takaishi, T. Kasahara, Y. Morita, and K. Ito. 1997. Eotaxin is a potent chemotaxin for human basophils. *Biochem. Biophys. Res. Commun. 231:365.*
4. Gonzalo, J. A., C. M. Lloyd, L. Kremer, E. Finger, C. Martinez-A, M. H. Siegelman, M. Cybulsky, and J. C. Gutierrez-Ramos. 1996. Eosinophil recruitment to the lung in a murine model of allergic inflammation; the role of T cells, chemokines, and adhesion receptors. *J. Clin. Invest. 98:2332.*
5. Rothenberg, M. E., A. D. Luster, C. M. Lilly, J. M. Drazen, and P. Leder. 1995. Constitutive and allergen-induced expression of eotaxin mRNA in the guinea pig lung. *J. Exp. Med. 181:1211.*
6. Lamkhioued, B., P. M. Renzi, S. Abi-Younes, E. A. Garcia-Zepeda, Z. Allakhverdi, O. Ghaffar, M. E. Rothenberg, A. D. Luster, and Q. Hamid. 1997. Increased expression of eotaxin in brochoalveolar lavage and airway of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J. Immunol. 159:4593.*
7. Mattoli, S., M. A. Stacey, G. Sun, A. Bellini, and M. Marini. 1997. Eotaxin expression and eosinophilic inflammation in asthma. *Biochem. Biophys. Res. Commun. 236:299.*
8. Stellato, C., L. A. Beck, G. A. Gorgone, D. Proud, T. J. Schall, S. J. Ono, L. M. Lichtenstein, and R. P. Schleimer. 1995. Expression of the chemokine RANTES by a human bronchial epithelial cell line: modulation by cytokines and glucocorticoids. *J. Immunol. 155:410.*
9. Beck, L. A., C. Stellato, L. D. Beall, T. J. Schall, D. Leopold, C. A. Bickel, F. Baroody, B. S. Bochner, and R. P. Schleimer. 1996. Detection of the chemokine RANTES and endothelial adhesion molecules in nasal polyps. *J. Allergy Clin. Immunol. 98:766.*
10. Garcia-Zepeda, E. A., M. E. Rothenberg, R. T. Ownbey, J. Celestin, P. Leder, and A. D. Luster. 1996. Human eotaxin is a specific chemoattractant for eosinophil

cells and provides a new mechanism to explain tissue eosinophilia. *Nat. Med. 2:449.*
11. Li, L., Y. Xia, A. Nguyen, L. Feng, and D. Lo. 1998. Th2-induced eotaxin expression and eosinophilia coexist with Th1 responses at the effector stage of lung inflammation. *J. Immunol. 161:3128.*
12. Maclean, J. A., R. Ownbey, and A. D. Luster. 1996. T-cell-dependent regulation of eotaxin in antigen-induced pulmonary eosinophilia. *J. Exp. Med. 184:1461.*
13. Lilly, C. M., H. Nakamura, H. Kesseiman, C. N. Anderson, K. Asano, E. A. Garcia-Zepeda, M. E. Rothenberg, J. M. Drazen, and A. D. Luster. 1997. Expression of eotaxin by human lung epithelial cells. *J. Clin. Invest. 99:1767.*
14. Mochizuki, M., J. Bartels, A. I. Mallet, E. Christophers, and J. M. Schroder. 1998. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J. Immunol. 160:60.*
15. Stellato, C., S. Matsukura, A. Fal, J. White, L. A. Beck, D. Proud, and R. P. Schleimer. 1999. Differential regulation of epithelial-derived C-C chemokine expression by IL-4 and the glucocorticoid budesonide. *J. Immunol. 163: 5624.*
16. Steffen, M., M. Abboud, G. K. Potter, X. P. Yung, and M. A. Moore. 1989. Presence of tumor necrosis factor or a related factor in human basophil/mast cells. *Immunology 66:445.*
17. Vecchiarelli, A., A. Siracusa, E. Cenci, M. Puliti, and G. Abbritti. 1992. Effect of corticosteroid treatment on interleukin-1 and tumor necrosis factor secretion by monocytes from subjects with asthma. *Clin. Exp. Allergy 22:365.*
18. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. Exp. Med. 155:914.*
19. Brown, M. A., J. H. Pierce, C. J. Watson, J. Falco, J. N. Ihle, and W. E. Paul. 1987. B cell stimulatory factor-1/interlukin-4 mRNA is expressed by normal and transformed mast cell. *Cell 50:809.*
20. Seder, R. A., W. E. Paul, S. S. Ben, G. S. LeGros, S. A. Kagey, F. D. Finkelman, J. H. Pierce, and M. Plaut. 1991. Production of interleukin-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. *Int. Arch. Allergy Appl. Immunol. 94:137.*
21. Baldwin, A. S. J. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol. 14:649.*
22. Kennedy, T., A. J. Ghio, W. Reed, J. Samet, J. Zagorski, J. Quay, J. Carter, L. Dailey, J. R. Hoidal, and R. B. Devlin. 1998. Copper-dependent inflammation and nuclear factor κB activation by particulate air pollution. *Am. J. Respir. Cell Mol. Biol. 19:366.*
23. Manni, A., J. Kleimberg, V. Ackerman, A. Bellini, F. Patalano, and S. Mattoli. 1996. Inducibility of RANTES mRNA by IL-1β in human bronchial epithelial cells is associated with increased NF-κB DNA activity. *Biochem. Biophys. Res. Commun. 220:120.*
24. Hou, J., U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight. 1994. An interleukin-4-induced transcription factor: IL-4 STAT. *Science 265: 1701.*
25. Mikita, T., D. Campbell, P. Wu, K. Williamson, and U. Schindler. 1996. Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. *Mol. Cell. Biol. 16:5811.*
26. Guo, F. H., K. Uetani, S. J. Haque, B. R. G. Williams, R. A. Dweik, F. B. J. M. Thunnisesen, W. Calhoun, and S. C. Erzurum. 1997. Interferon γ and interleukin 4 stimulate prolonged expression of inducible nitric oxide synthase in human airway epithelium through synthesis of soluble mediators. *J. Clin. Invest. 100:829.*
27. Reddel, R. R., Y. Ke, B. I. Gerwin, M. G. McMenamin, J. F. Lechner, R. T. Su, D. E. Brash, P. B. Park, J. S. Rhim, and C. C. Harris. 1988. Transformation of human bronchial epithelial cell by infection with SV40 or adenovirus-12 SV4-hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res. 48:1904.*
28. Siebert, P. D., A. Chenchik, D. E. Kellogg, K. A. Lukyanov, and S. A. Lukyanov. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res. 23:1087.*
29. Ponath, P. D., S. Qin, D. J. Ringler, I. C. Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J. A. Gonzaio, W. Newman, et al. 1996. Cloning of the human eosinophil chemoattractant, eotaxin. *J. Clin. Invest. 97:604.*
30. Schreiber, E., P. Matthias, M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with mini-extracts from a small number of cells. *Nucleic Acids Res. 17:6419.*
31. Bharvin, K., R. Patel, J. H. Pierce, and W. J. LaRochelle. 1998. Regulation of interleukin 4-mediated signaling by naturally occurring dominant negative and attenuated forms of human Stat6. *Proc. Natl. Acad. Sci. USA 95:172.*
32. Georas, S. N., J. E. Cumberland, T. F. Burke, R. Chen, U. Schindler, and V. Casolaro. 1998. Stat6 inhibits human interleukin-4 promoter activity in T cells. *Blood 92:4529.*
33. Miura, K., J. T. Schroeder, W. C. Hubbard, and D. W. M. Jr. 1999. Extracellular signal-regulated kinases regulate leukotriene C4 generation, but not histamine release or IL-4 production from human basophils. *J. Immunol. 162:4198.*
34. Garcia-Zepeda, E. A., M. E. Rothenberg, S. Weremowicz, M. N. Sarafi, C. C. Morton, and A. D. Luster. 1997. Genomic organization, complete sequence, and chromosomal location of the gene for human eotaxin (SCYA11), an eosinophil-specific CC chemokine. *Genomics 41:471.*
35. Hein, H., C. Schluster, R. Kulke, E. Christophers, J. M. Schroder, and J. Bartels. 1997. Genomic organization, sequence, and transcriptional regulation of the human eotaxin gene. *Biochem. Biophys. Res. Commun. 237:537.*
36. Iademarco, M. F., J. J. McQuillan, G. D. Rosen, and D. C. Dean. 1992. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). *J. Biol. Chem. 267:16323.*
37. Ledebur, H., and T. Parks. 1995. Transcriptional regulation of the ICAM-1 gene by inflammatory cytokines in human endothelial cells: essential roles of a variant NF-κB site and p65 homodimers. *J. Biol. Chem. 270:933.*
38. Ping, D., G. H. Boekhoudt, E. M. Rogers, and J. M. Boss. 1999. Nuclear factor-κB p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene. *J. Immunol. 162:727.*
39. Stein, B., and A. S. B. Jr. 1993. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. *Mol. Cell. Biol. 13:7191.*
40. Xie, Q. W., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem. 269:4705.*
41. Kaplan, M. H., U. Schindler, S. T. Smiley, and M. J. Grusby. 1996. STAT6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity 4:313.*
42. Shimoda, K. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature 380:630.*
43. Takeda, K. 1996. Essential role of Stat6 in IL-4 signalling. *Nature 380:627.*
44. Chen, F. E., D. B. Huang, Y. Q. Chen, and G. Ghosh. 1998. Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA. *Nature 391: 410.*
45. Seidel, H. M., L. H. Milocco, P. Lamb, J. J. E. Darnell, R. B. Stein, and J. Rosen. 1995. Spacing of palindromic half sites as a determinant of selective STAT (signal transducers and activators of transcription) DNA binding and transcription activity. *Proc. Natl. Acad. Sci. USA 92:3041.*
46. Guo, D., J. D. Dunbar, C. H. Yang, L. M. Pfeffer, and D. B. Donner. 1998. Induction of Jak/STAT signaling by activation of the type 1 TNF receptor. *J. Immunol 160:2742.*
47. Lugli, S. M., N. Feng, M. H. Heim, M. Adam, B. Schnyder, H. Etter, M. Yamage, H. P. Eugster, R. A. Lutz, G. Zurawski, et al. 1997. Tumor necrosis factor α enhances the expression of the interleukin (IL)-4 receptor α-chain on endothelial cells increasing IL-4 or IL-13-induced Stat6 activation. *J. Biol. Chem. 272:5487.*
48. Shen, C. H., and J. Stavnezer. 1998. Interaction of STAT6 and NF-κB: direct association and synergistic activation of interleukin-4-induced transcription. *Mol. Cell. Biol. 18:3395.*
49. Adcock, I. M., C. R. Brown, C. M. Gelder, H. Shirasaki, M. J. Peters, and P. J. Barnes. 1995. Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. *Am. J. Physiol. 268:C331.*
50. Kiehntopf, M., F. Herrmann, and M. A. Brach. 1995. Functional NF-IL-6/CCAAT enhancer-binding protein is required for tumor necrosis factor α-inducible expression of the granulocyte colony-stimulating factor (CSF), but not the granulocyte/macrophage CSF or interleukin 6 gene in human fibroblasts. *J. Exp. Med. 181:793.*
51. Lundgren, M., C. Larsson, A. Femino, M. Xu, J. Stavnezer, and E. Severinson. 1994. Activation of the Ig germ-line γ1 promoter: involvement of C/Enhancer-binding protein transcription factors and their possible interaction with an NF-IL-4 site. *J. Immunol. 153:2983.*
52. Benett, B. L., R. Cruz, R. G. Lacson, and A. M. Mannings. 1997. Interleukin-4 suppression of tumor necrosis factor α-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-κB. *J. Biol. Chem. 272:10212.*
53. Iciek, L. A., S. A. Delphin, and J. Stavnezer. 1997. CD40 cross-linking induces Ig epsilon germline transcripts in B cells via activation of NF-κB: synergy with IL-4 induction. *J. Immunol. 158:4769.*
54. Messner, B., A. M. Stutz, B. Albrecht, S. Peiritsch, and M. Woisetschlager. 1997. Cooperation of binding sites for STAT6 and NF-κB/Rel in the IL-4-induced up-regulation of the human IgE germline promoter. *J. Immunol. 159:3330.*
55. Isalan, M., Y. Choo, and A. Klug. 1997. Synergy between adjacent zinc fingers in sequence-specific DNA recognition. *Proc. Natl. Acad. Sci. USA 94:5617.*
56. Chen, X., U. Vinkemeier, Y. Zhao, D. Jeruzalmi, J. J. E. Darnell, and J. Kuriyan. 1998. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell 93:827.*
57. Wotton, D., J. Ghysdael, S. Wang, N. A. Speck, and M. J. Owen. 1994. Cooperative binding of Ets-1 and core binding factor to DNA. *Mol. Cell. Biol. 14:840.*
58. Yang, L., L. Cohn, D. H. Zhang, R. Homer, A. Ray, and P. Ray. 1998. Essential role of nuclear factor κB in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med. 188:1739.*
59. Akimoto, T., F. Numata, M. Tamura, Y. Takata, N. Higashida, T. Takashi, K. Takeda, and S. Akira. 1998. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J. Exp. Med. 187:1537.*
60. Kuperman, D., B. Schofield, M. W. Karp, and M. J. Grusby. 1998. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med. 187:939.*