Interleukin-4 (IL-4), an immunoregulatory cytokine secreted from activated T-helper 2 lymphocytes, eosinophils, and mast cells, stimulates the expression of a number of immune system genes via activation of the transcription factor, STAT6. However, IL-4 can concomitantly suppress the expression of other immune-related gene products, including k light chain, FcγRI, IL-8, and E-selectin. We demonstrate that IL-4 activates STAT6 in human vascular endothelial cells and that two STAT6 binding sites are present in the promoter of the E-selectin gene. IL-4-induced STAT6 binding does not activate E-selectin transcription but instead suppresses tumor necrosis factor α-induced expression of the E-selectin gene. STAT6 was found to compete for binding to a region in the E-selectin gene promoter containing overlapping STAT6 and NF-κB binding sites, effectively acting as an antagonist of NF-κB binding and transcriptional activation. This novel mechanism for IL-4-mediated inhibition of inflammatory gene expression provides an example of a STAT factor acting as a transcriptional repressor rather than an activator.

Interleukin-4 Suppression of Tumor Necrosis Factor α-stimulated E-selectin Gene Transcription Is Mediated by STAT6 Antagonism of NF-κB

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The abbreviations used are: IL, interleukin; TH2, T-helper 2; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; HUVEC, human umbilical vein endothelial cell; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.
both the phosphorylation and proteolytic degradation of IκB and results in the translocation of NF-κB to the nucleus where it can bind to specific promoter sequence elements and induce gene transcription (26). In vascular endothelium, IL-4 differentially regulates the expression of cell adhesion molecules such as E-selectin and VCAM-1, and proinflammatory cytokines such as IL-6 and IL-8 (16–18). IL-4 augments the expression of VCAM-1 and IL-6 but concomitantly suppresses the expression of E-selectin and IL-8 (22, 27, 28), thereby modulating leukocyte recruitment to sites of inflammation (19). Treatment with IL-4 alone increases VCAM-1 protein levels via stabilization of the VCAM-1 mRNA (30). However, the mechanisms by which IL-4 suppresses E-selectin and IL-8 levels is unknown. The studies we describe in this report were designed to explore a possible role for STAT6 in the IL-4 modulation of endothelial E-selectin gene expression.

MATERIALS AND METHODS

Culture of Human Umbilical Vein Endothelial Cells—Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured as described (29). Briefly, HUVECs were plated in tissue culture dishes pretreated with 0.1% gelatin and grown in medium M199 (Life Technologies, Inc.) containing 20% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine (Life Technologies, Inc.) containing 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 20 ng/ml endothelial cell growth supplement (Collaborative Research, Medford, MA), 10 units/ml heparin (Sigma), and 100 units/ml penicillin G with 100 μg/ml of streptomycin sulfate (Life Technologies, Inc.). Cells used in experiments were from passages 3 to 5. Cells grown to confluence in 96-well microtitre plates. Cytokine was added as a 10-μl addition to the well medium. Treatments were either TNFα (300 units/ml; specific activity, 2 × 10⁹ units/mg) or IL-4 (10 ng/ml) (Genzyme, Cambridge, MA) or both as described in the text. At the end of the incubation period, cells were washed once with phosphate-buffered saline (PBS) and incubated with freshly prepared 4% paraformaldehyde solution, pH 7, for 60 min. Plates were then washed once with PBS, blocked overnight at 4°C with 2% bovine serum albumin in PBS, washed once with PBS, and incubated with 1 μg/ml primary antibody in 0.1% bovine serum albumin in PBS at 37°C for 2 h. Monoclonal antibody to VCAM-1 (CL40) was from Pharmacia & Upjohn, whereas monoclonal antibody to E-selectin was from R & D Systems (BBAI, Minneap. Minn.). After incubation with primary antibody, the cells were washed three times with 0.05% Tween 20 in PBS, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:500) (Organon Teknika Corp., West Chester, PA) in 0.1% bovine serum albumin in PBS at 37°C for 1 h, washed three times with 0.05% Tween 20 in PBS, and washed once with PBS. The cells were then incubated in chromogenic substrate (1 mg/ml 4-nitrophenyl phosphate in 10 μg/ml sodium carbonate, pH 9.8) at 37°C for 45 min and absorbance was measured at 405 nm using a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA). The results are presented as mean ± standard deviation of quadruplicate samples. Statistical significance was determined using two way analysis of variance.

Preparation of Nuclear Extracts—HUVECs were treated with TNFα (300 units/ml; specific activity, 2 × 10⁹ units/mg) or IL-4 (10 ng/ml) or both for 30 min. Cells were subjected to treatment with trypsin and pelleted by centrifugation at 1000 × g for 5 min. Nuclear proteins were isolated as described previously (29).

Western Blotting—Nuclear extracts (125 μg) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel, and the fractionated proteins were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA) using a Multiphor II semi-dry blotting device (Pharmacia Biotech Inc.). The membrane was blocked with 4% nonfat milk powder in PBS with 0.05% Tween 20 (PBS-T), incubated with 0.1 μg/ml rabbit polyclonal antibody to STAT6 (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-T, washed, and then incubated with a polyclonal donkey anti-rabbit IgG antibody (1:2000) conjugated with horseradish peroxidase (Amersham Corp.). After extensively washing the membrane in PBS-T, the membrane was exposed to the chemiluminescent substrate (ECL detection system, Amersham Corp.), and the membrane was subjected to autoradiography with Hyperfilm MP (Amersham Corp.).

Electrophoretic Mobility Shift Assay—Oligonucleotides were end-labeled with [γ-32P]ATP and T4 polynucleotide kinase as single strands prior to annealing to form double stranded target molecules. The NF-κB consensus DNA, 5′-AGTGAGGGGACTTTCCAGGC-3′, was pur chased as a double stranded oligonucleotide from Promega (Madison, WI). The oligonucleotide sequences used for E-selectin and FcγRII are given under “Results.” For each assay, 10 μg of nuclear protein extract was incubated with 35 fmol of 32P-labeled oligonucleotide probe in binding buffer (4% glycerol, 10 mM Tris·Cl, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 50 mM Tris·Cl, pH 7.5) for 30 min at room temperature. Competition studies were performed by the addition of a 50-fold molar excess of unlabeled oligonucleotide to the binding reaction. Supershifting experiments were performed by the addition of antibody to the binding reaction for 20 min following the normal 20-min binding reaction. Rabbit polyclonal antibodies to p50 and p65 from Santa Cruz Biotechnology (Santa Cruz, CA). Resultant protein-DNA complexes were resolved on native 6% polyacrylamide gels using a 0.5 × TBE buffer (0.1 M Tris·Cl, 90 mM boric acid, 1 mM EDTA, pH 8.4). The gels were prerun for 45 min at 150 V, and the samples were then electrophoresed at 250 V for 2–3 h at 4°C, dried on paper under vacuum, and subjected to autoradiography.

Promoter Reporter Assays—HUVECs were co-transfected with pCMVβ (Clontech, Palo Alto, CA), a mammalian vector containing the β-galactosidase reporter driven by the cytomegalovirus promoter, and pE-luc, a mammalian vector containing 816 base pair of the E-selectin promoter (−9 to −870 base pairs from translation start site) inserted upstream of the luciferase reporter plasmid pGL2 (Promega, Madison, WI). Site-directed mutagenesis of pE-luc was performed using Pfu DNA polymerase (Stratagene) extension of both plasmid strands from mutated primers. The sense primers were, 5′-GCATCGTGGATAT(T)CCGGGAAG-3′ where the Ts in parentheses were changed to Cs to construct two separate point mutants. Clones were sequenced and shown to contain only the introduced mutation. The transfection procedure used Lipofectin reagent as outlined in the manufacturer’s instructions (Life Technologies, Inc.). Briefly, cells were seeded into gelatin coated 6-well plates and allowed to grow to 60% confluency. Cells were transfected with 6 μl of Lipofectin and 500 ng of each vector in 800 μl of serum-free medium for 3.5 h and then incubated in normal medium for 24–48 h. In experiments where total cytokine treatment was for 5 h, the cytokine was added 48 h after transfection. In experiments involving cytokine pretreatment, the cytokine was added 30 h after transfection. Following cytokine treatment cells were washed once in ice-cold PBS, solubilized by incubation in 200 μl of reporter lysis buffer for 15 min (Promega, Madison, WI), transferred to a 96-well plate, and centrifuged to pellet cellular debris, and the supernatant was stored at −80°C. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI), and β-galactosidase activity was determined by the Luminescent β-galactosidase Genetic Reporter System (ChemiGen, CA). Activities were quantified in a model ML1000 Luminometer with version 3.993 software (Dynatech, Chantilly, VA). Final E-selectin-luciferase activities were normalized against β-galactosidase activity.

Immunofluorescent Staining of Transcription Factors—HUVECs were grown to confluence on gelatin-coated coverslips, treated with TNFα (300 units/ml; specific activity, 2 × 10⁹ units/mg) or IL-4 (10 ng/ml) or both for 48 h with 4% paraformaldehyde solution, and paraffin wax infiltration and a fluorescein excitation filter and photographed on Kodak Gold 400 print film.

Isolation of RNA and Northern Blotting—Total RNA was isolated from HUVECs using the reagent RNeasy (Tel-Test, Inc., Friendswood, TX) based on the procedure of Chomczynski and Sacchi (30). RNA was resuspended in 15 μl of 1 mM EDTA and quantified by absorbance at 260 nm. Total RNA was size fractionated on a 1% agarose 0.6 M formaldehyde gel (31). After electrophoresis the gel was blotted onto Hy bond N+ membrane (Amersham Corp.) using the manual capillary blot transfer system (Life Technologies, Inc.), and the RNA was cross-linked to the membrane by UV irradiation in a Stratalinker 2400 (Stratagene, La Jolla, CA). CDNA’s of approximately 500 base pairs in length were isolated by reverse transcription-polymerase chain reaction from HUVECs using the primer pair 5′-TGGCTCTACACACCCTC-3′ and 5′-CAGGCCTCTCAGTGCAC-3′ for human E-selectin (32) and 5′-TTG-
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CAGCTTCTCAAGCT-3' and 5'-ATCTCCAATGACAGGAG-3' for human VCAM-1 (33). A glyceraldehyde-3-phosphate dehydrogenase cDNA was isolated from an amplitex kit (Clontech, Palo Alto, CA). cDNA probes were prepared by random prime labeling with [a-32P]dCTP (3000 Ci/mmol) using the Prime-It II labeling kit (Stratagene, La Jolla, CA).

Statistical Analysis—Group means and standard deviations were analyzed using one-way analysis of variance.

RESULTS

To establish that IL-4 can differentially modulate cytokine-induced E-selectin and VCAM-1 levels, we examined the cell surface expression of E-selectin and VCAM-1 in HUVECs stimulated with TNFα in the presence or the absence of IL-4 (Fig. 1). Treatment with IL-4 alone for 5 or 23 h had no effect on E-selectin expression (Fig. 1A, lanes 2 and 3). However, consistent with previous reports (28), treatment with IL-4 alone significantly increased VCAM-1 levels above control (Fig. 1B, lanes 2 and 3). Treatment with TNFα resulted in significant increase in E-selectin and VCAM-1 cell surface levels (Fig. 1A, lanes 4). IL-4 treatment in conjunction with TNFα resulted in an augmentation of VCAM-1 protein levels and a significant, although not complete, inhibition of E-selectin expression. Of note, although no pretreatment was necessary to observe the IL-4 enhancement of VCAM-1 expression, pretreatment was required for inhibition of E-selectin.

The IL-4-dependent increase in VCAM-1 levels is known to result from a stabilization of VCAM-1 mRNA (28). We therefore explored whether treatment with IL-4 also affected E-selectin mRNA stability. IL-4 treatment augmented the levels of VCAM-1 mRNA after 4 h and stabilized VCAM-1 transcripts over the following 12 h as previously reported. In contrast, IL-4 treatment had no effect on the half-life of E-selectin mRNA (t1⁄2 = 5.5 h, t1⁄2 + IL-4 = 6 h) but did result in a 22% decrease in the levels of steady state E-selectin mRNA at 4 h (data not shown). Therefore, IL-4-dependent stabilization of mRNA is specific for VCAM-1 and was not found to occur with the mRNA for another cell adhesion molecule, namely E-selectin. The observed decrease in E-selectin mRNA levels suggested that the IL-4 effect on E-selectin expression was being mediated at the level of transcription. To demonstrate this further we examined the effect of IL-4 on the cytokine-induced transcriptional activation of the E-selectin promoter, using an E-selectin promoter-luciferase reporter gene construct. HUVEC monolayers were cotransfected with a constitutively expressed β-galactosidase reporter gene construct and 861 base pairs of the E-selectin promoter. Transfected HUVECs were examined for E-selectin promoter activity using a promoter-reporter assay. Cells were treated with medium alone (lane 1), IL-4 for 5 h (lane 2), TNFα for 5 h (lane 3), TNFα and IL-4 for 5 h (lane 4), 20 min IL-4 pretreatment followed by TNFα and IL-4 for 5 h (lane 5). The values represent the means ± S.D. of six replicates and are representative of five independent experiments. p, p < 0.001 compared with TNFα-treated cells (lane 3).

FIG. 1. IL-4 modulates the TNFα-induced cell surface expression of E-selectin and VCAM-1 on HUVECs. HUVECs were treated with TNFα (300 units/ml) and/or IL-4 (10 ng/ml) and assayed by ELISA for E-selectin (A) or VCAM-1 (B) cell surface expression. Samples were treated with medium alone (lane 1), IL-4 for 5 h (lane 2), IL-4 for 23 h (lane 3), TNFα for 5 h (lane 4), TNFα and IL-4 for 5 h (lane 5), 18 h IL-4 pretreatment followed by TNFα and IL-4 for 5 h (lane 6), and 20 min IL-4 pretreatment followed by TNFα and IL-4 for 5 h (lane 7). The values represent the means ± S.D. of four replicates and are representative of three independent experiments. †, significantly different from untreated cells (p < 0.01); * significantly different from TNFα-treated cells (p < 0.01).

FIG. 2. IL-4 treatment inhibits TNFα-induced activity of the E-selectin promoter. Transfected HUVECs were examined for E-selectin promoter activity using a promoter-reporter assay. Cells were treated with medium alone (lane 1), IL-4 for 5 h (lane 2), TNFα for 5 h (lane 3), TNFα and IL-4 for 5 h (lane 4), 20 min IL-4 pretreatment followed by TNFα and IL-4 for 5 h (lane 5). The values represent the means ± S.D. of six replicates and are representative of five independent experiments. #, p < 0.001 compared with TNFα-treated cells (lane 3).
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In leukocytes, IL-4 has been shown to activate STAT6, a key component of the IL-4-dependent transcription factor, IL-4 STAT (7). We therefore examined whether STAT6 was present in endothelial cells and activated in response to IL-4 treatment. Nuclear extracts from HUVECs treated with TNFα or IL-4 were examined for immunoreactivity with a polyclonal STAT6 antibody in a Western blot. Immunofluorescent staining of STAT6 and p65 in HUVECs. Cells were treated with TNFα (300 units/ml) or IL-4 (10 ng/ml) for 30 min, and nuclear proteins were analyzed for immunoreactivity with a monoclonal antibody to STAT6 or p65 and visualized with fluorescein isothiocyanate immunofluorescence.

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A previous report has shown that IL-4 directly inhibits the activation of NF-κB in monocytes but not fibroblasts (36). To investigate this mechanism we treated HUVECs with TNFα for 20 min, with and without a 20-min IL-4 pretreatment, and examined nuclear extract DNA binding activity to STAT6 and NF-κB oligonucleotides (Fig. 4). STAT6 binding activity was only observed in cell extracts from IL-4-treated cells. We observed a minor decrease in NF-κB DNA binding activity from cells treated with TNFα and IL-4 compared with cells treated with TNFα alone (see legend to Fig. 4). Therefore, IL-4 did not appear to markedly inhibit NF-κB activation in HUVECs.

We hypothesized that STAT6 binding sites may be present in the E-selectin gene promoter and that STAT6 binding may mediate the IL-4-induced inhibition of E-selectin expression. Computer analysis of the E-selectin promoter revealed two potential STAT6 recognition sequences (TTCN3–4GAA) (16) at positions –112 to –112 (E-selectin A) and –41 to –50 (E-selectin B) relative to the transcription initiation site (Fig. 5A). Oligonucleotides corresponding to these sites were synthesized, and the ability of nuclear proteins to interact with these two putative sequence elements was examined (Fig. 5B). IL-4-inducible complexes of identical electrophoretic mobility were observed binding to both of the E-selectin oligonucleotides and to an oligonucleotide containing a known STAT6 binding site from the FcyRI gene (7). The IL-4-inducible complex formed with the FcyRI oligonucleotide was diminished in the presence of STAT6 antibody, confirming that the DNA-protein complex contained STAT6 (Fig. 5B, lane 3). The presence of excess FcyRI oligonucleotide resulted in the complete loss of the IL-4-induced E-selectin A and B complexes, demonstrating that the factor interacting with the E-selectin sites was the same as that binding to the FcyRI sequence (lanes 6 and 10). Addition of STAT6 antibody to the binding reactions inhibited complex formation similar to that observed with FcyRI (lanes 7 and 11). Therefore, STAT6 specifically recognizes two sites within the
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E-selectin gene promoter, and STAT6 binding to these sites is induced by IL-4 treatment.

To determine whether IL-4-induced activation of STAT6 correlated directly with the observed inhibition of E-selectin expression, we used EMSA and ELISA analyses to compare the dose dependence of IL-4 activation of STAT6 (Fig. 6A) and inhibition of E-selectin cell surface protein levels (Fig. 6B). The dose-dependent concentration range of STAT6 nuclear localization and DNA binding activity to the E-selectin A site was 0.1–10 ng/ml IL-4. Similarly, IL-4 inhibition of TNFα-induced E-selectin expression was observed at concentrations between 0.1 and 10 ng/ml IL-4, with maximal inhibition at 10 ng/ml.

Inspection of the sequences surrounding the E-selectin A site revealed that this STAT6 recognition sequence overlaps two adjacent, well characterized NF-κB binding sites critical for TNFα-induced transcriptional activation (Fig. 7A) (34). This observation suggested that binding of STAT6 to this site might antagonize NF-κB binding and account for the observed IL-4 suppression of TNFα-induced E-selectin expression. To explore this hypothesis, we synthesized an extended oligonucleotide containing both the NF-κB sites and the STAT6 site, as shown in Fig. 6A, and examined the binding of TNFα- and IL-4-activated nuclear proteins to this sequence (Fig. 7B). Consistent with earlier experiments, nuclear extracts from cells treated with IL-4 induced the formation of a complex that was inhibited by the addition of unlabeled FcγRI oligonucleotide or STAT6 antibody (Fig. 7B, lanes 2–4). Nuclear extracts from cells treated with TNFα alone induced a distinct complex of different mobility than that identified as containing STAT6 (Fig. 7B, lane 5). Formation of this complex was inhibited by the addition of unlabeled NF-κB binding oligonucleotide or antibodies to the NF-κB component proteins, p50 and p65 (Fig. 7B, lanes 6 and 7). When nuclear extracts from cells treated with both TNFα and IL-4 were analyzed, both the NF-κB and STAT6 complexes were present in the same binding reaction (Fig. 7B, lane 8). No novel complexes were observed, suggesting that NF-κB and STAT6 cannot bind to the same site simultaneously. Addition of unlabeled FcγRI exclusively diminished STAT6 binding, whereas unlabeled NF-κB oligonucleotide prevented formation of NF-κB complex only (Fig. 7B, lanes 9–10).

Similarly, addition of STAT6 antibody resulted in a loss of the STAT6 containing complex without affecting the NF-κB complex, whereas antibody to p50 and p65 resulted in the loss of the NF-κB complex without affecting the STAT6 complex (Fig. 7B, lanes 11–12). Therefore, NF-κB and STAT6 appear to bind a shared region of the E-selectin promoter in a manner that is mutually exclusive and consequently antagonistic.

To demonstrate this transcription factor antagonism in vivo, we performed site-directed mutagenesis on the E-selectin promoter-reporter construct. Two point mutant constructs were generated that changed the STAT6 binding site but retained the NF-κB binding consensus sequence of the upstream site. According to a previous study, mutant-1 should abolish STAT6 binding, and mutant-2 should retain the STAT6 consensus sequence (8). In a promoter-reporter assay (Fig. 8) the control E-selectin luciferase construct showed enhanced luciferase activity in the presence of TNFα that was inhibited 50% with an IL-4 pretreatment (lanes 1–3) as shown in an earlier experiment (Fig. 2). Mutant-1 showed an unexpected partial loss of TNFα stimulation, suggesting that this point mutation does affect NF-κB binding even though the introduced mutation conformed to the theoretical consensus sequence. However, no additional inhibition was observed with IL-4, suggesting that STAT6 binding at this site is responsible for the inhibitory effect observed (Fig. 8, lanes 4–6). A second mutant-1 isolate exhibited the same activity profile (data not shown). Mutant-2

Fig. 5. STAT6 binds to two sites in the E-selectin gene promoter. A lists the three oligonucleotides used in the EMSA analyses. The specific STAT6 binding sites are underlined, and consensus nucleotides are capitalized. The FcγRI oligonucleotide was used as a STAT6 binding control (7). B, EMSA analysis of STAT6 binding to FcγRI (lanes 1–3), E-selectin site A (lanes 4–7), and E-selectin B (lanes 8–11) oligonucleotide probes. Nuclear extract was prepared from HUVECs treated with medium alone (lanes 1, 4, and 8) or with 10 ng/ml IL-4 for 30 min. Binding was competed with a STAT6 antibody (lanes 3, 7, and 11) or with excess unlabeled FcγRI oligonucleotide (lanes 6 and 10). NS, nonspecific binding; Ab, antibody.

Fig. 6. IL-4 dose dependence of STAT6 activation and inhibition of E-selectin cell surface protein. A, nuclear extracts were isolated from HUVECs treated for 30 min with a range of IL-4 concentrations as shown. Extracts were mixed with the E-selectin A oligonucleotide probe and analyzed in an EMSA. B, HUVECs were pretreated with a range of IL-4 concentrations for 30 min prior to the addition of TNFα for 4 h. E-selectin cell surface protein levels were determined in an ELISA assay. The values represent the percentage of inhibition of untreated cells for quadruplicate samples. The result is representative of two independent experiments.
NF-κB presses E-selectin protein levels via the transcription factor which TNFα activates by binding to the STAT6 binding site and the two NF-κB sites. This results in the transcriptional activation of E-selectin expression by proinflammatory mediators such as TNFα, but has little effect on eosinophil adhesion (42). E-selectin inhibits neutrophil attachment to activated endothelial surface (41). Indeed, monoclonal antibodies to E-selectin inhibit initial attachment and rolling of neutrophils (40). Intravital microscopy of leukocytes rolling in E-selectin-deficient mice showed TNFα stimulation similar to that observed for IL-4, confirming that this mutation does not abolish STAT6 binding (Fig. 8, lanes 7–9).

**DISCUSSION**

IL-4 has been shown in vivo to retard the infiltration of neutrophils and monocytes and to enhance the recruitment of lymphocytes and eosinophils to sites of inflammation (3, 37). Selective leukocyte recruitment is a feature of inflammatory diseases such as asthma, which are characterized by an eosinophilic infiltrate and high levels of IL-4 (6). Evidence suggests that the mechanisms whereby IL-4 exerts this effect are diverse but include the augmentation of VCAM-1, an important ligand for eosinophil adhesion but not required for the recruitment of neutrophils (38); the inhibition of IL-8 secretion, a major neutrophil chemoattractant (39); and the suppression of IL-8 production, a ligand for eosinophil adhesion but not required for the recruitment of eosinophils (40). IL-4 is shown to promote up to 50% of the total NF-κB activity in the presence of TNFα and IL-4. Shown are control (lanes 1–3, TCCCGGGAAT), mutant 1 (lanes 4–6, CTCCGGAAT), and mutant 2 (lanes 7–9, TCCCGGGAAT). Transfected cells were left untreated (lanes 1, 4, and 7), treated with TNFα for 5 h (lanes 2, 5, and 8), or pre-treated with IL-4 for 20 min followed by TNFα for 5 h (lanes 3, 6, and 9). The values represent the means ± S.D. of six replicates and are representative of three independent experiments. #, p < 0.001; *, p < 0.005 compared with TNFα-treated cells. Ab, antibody.

STAT6 and NF-κB compete for binding to the E-selectin A site. The STAT6 binding site and the two NF-κB sites are shown with the labeled black bars. Consensus nucleotides for the STAT6 site are capitalized. B, binding of STAT6 or NF-κB to the E-selectin A site is mutually exclusive. EMSA analysis using the extended E-selectin A oligonucleotide shown in A and HUVEC nuclear extracts from cells treated with IL-4 (10 ng/ml), TNFα (300 units/ml), or IL-4 and TNFα for 30 min as labeled in the figure. Reactions were competed with FcγRI, NF-κB consensus oligonucleotide, antibody to STAT6, or the p50 and p65 subunits of NF-κB. *treat., treatment.
presumably because it is unable to promote the formation of an effective transcriptional complex. This is interesting because a STAT6-containing transcription factor has been shown to activate the FcRMIIB/CD23, class II major histocompatibility complex, and mouse C domain of immunoglobulin heavy chain genes in leukocytes (8, 9). It will be important to compare the composition of STAT6 transcription factor complexes in endothelial cells and monocytes. It will also be valuable to identify STAT6 genes that are transcriptionally activated by STAT6 in endothelial cells so as to define additional nuclear factors required for STAT6-mediated transcription.

Activation and translocation of STAT6 by IL-4 is rapid, leading to STAT6 DNA binding activity within 30 min of exposure to cytokine. Although some inhibition of TNFα-induced E-selectin promoter activity was observed when TNFα and IL-4 were added simultaneously, the inhibitory effect of IL-4 was more pronounced when HUVECs were incubated with IL-4 for 20 min prior to TNFα treatment. This suggests that either the pathway of NF-κB activation is more rapid than that of STAT6 or that protein-DNA binding kinetics at the E-selectin A site in vivo favor binding of NF-κB so that elevated levels of STAT6 in the nucleus are required to effectively compete with NF-κB for binding. Our observation that the E-selectin A site is completely integrated within two adjacent NF-κB sites presents the possibility that conservation of the STAT6 recognition sequence might be a secondary consequence of conservation of the NF-κB sites. Although the decameric NF-κB consensus sequence varies considerably in many genes shown to be transcriptionally activated by NF-κB (26), we were unable to de-stray STAT6 activity without affecting NF-κB activity. Therefore, conservation of the STAT6 site within two potentially variable NF-κB sites could represent the selective retention of an important biological role for IL-4 on expression of the E-selectin gene. In this regard, Schindler and Baichwal (34) have shown that nucleotide changes to the key residues comprising the STAT6 binding site result in significant inhibition of NF-κB-mediated promoter activity. Therefore, the conservation and interaction of these three sites appears closely linked.

The mechanisms whereby IL-4 modulates the expression of E-selectin and VCAM-1 in endothelium highlights the fact that an individual cytokine may activate different intracellular signal transduction pathways. Although IL-4 treatment resulted in STAT6 activation and the consequent suppression of E-selectin gene transcription, we found no STAT6 binding sites in the VCAM-1 promoter. IL-4 accumulation of VCAM-1 expression is instead mediated via a poorly characterized pathway that results in the stabilization of VCAM-1 mRNA. This mechanism appears highly selective because it had no effect on E-selectin mRNA or glyceraldehyde-3-phosphate dehydrogenase mRNA stability (28). In addition, IL-4 has been shown to result in the phosphorylation of a 170-kDa substrate called 4PS (44). This protein is related by homology to insulin receptor substrate-1 and interacts with phosphatidylinositol-3 kinase. Therefore, IL-4 may activate at least three signal transduction pathways in a single cell type. Drug discovery efforts that focus upon exploiting these discrete signaling pathways may yield therapies with enhanced biological specificity and reduced side effects.

The purpose of this study was to examine the molecular mechanism whereby an immunomodulatory cytokine, IL-4, may act on E-selectin expression and thereby alter the inflammatory profile of diseases such as asthma. That E-selectin suppression is mediated by an antagonism of NF-κB reiterates the importance of this transcription factor in inflammatory disease. NF-κB has been shown to be activated in conditions as diverse as allergic airway inflammation (45), atherosclerosis (46), endotoxic shock (47), ischaemia-reperfusion injury (48), rheumatoid arthritis (49), restenosis (50), and sunburn (51). Furthermore, it is notable that many agents that exhibit anti-inflammatory properties have been shown to inhibit NF-κB action including glucocorticoids (52, 53), antioxidants (54), salicylates (55), glio toxin (56), flavonoids (57), as well as endoge nous mediators such as nitric oxide (58) and the immunomodu latory cytokine, IL-10 (59). We now show that IL-4 also exerts some of its immunomodulatory action by inhibiting the actions of NF-κB.
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