IL-4 and IL-13 Activate the JAK2 Tyrosine Kinase and Stat6 in Cultured Human Vascular Endothelial Cells Through a Common Pathway That Does Not Involve the γ_c Chain

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Abstract

IL-4 and IL-13 each act on human endothelial cells (ECs) to induce expression of vascular cell adhesion molecule-1. On hematopoietic cells, IL-4 responses may be mediated either through a pathway involving g_c, the common signaling subunit of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors, or through a g_c-independent pathway that may be alternatively activated by IL-13. We find that human ECs do not express g_c, as detected by indirect immunofluorescence and FACS® analysis or by a reverse transcription-PCR method. Like IL-4, IL-13 activates a protein tyrosine kinase that phosphorylates the IL-4R binding protein. In addition, we find that IL-4 and IL-13 each induce tyrosine phosphorylation of the JAK2 tyrosine kinase. Furthermore, both IL-4 and IL-13 induce binding of the Stat6 transcription factor to a consensus sequence oligonucleotide. We conclude that the IL-4 response of human ECs involves the IL-13 shared pathway that is independent of g_c, and uses JAK2-Stat6 signaling. (*J. Clin. Invest.* 1996. 98:604–609.) Key words: cytokines • inflammation • Janus kinases • transcription factors • vascular cell adhesion molecule-1

Introduction

IL-4 is an important mediator of allergic inflammatory responses characterized by eosinophil and lymphocyte recruitment. The mechanism of action may, in part, involve responses of vascular endothelium. For example, IL-4 causes a small increase in the expression of endothelial vascular cell adhesion molecule-1 (VCAM-1, CD106)[1] and markedly augments VCAM-1 expression induced by TNF or IL-1 (1–6). These changes favor the binding of eosinophils and T cells to endothelial cells (ECs). In addition, IL-4–treated ECs secrete the chemokine MCP-1 (7) and selectively stimulate transendothelial migration of eosinophils in vitro (8). The effects of IL-4 on EC differ from those on hematopoietic cell types in that IL-4 is not an endothelial mitogen or survival factor.

IL-13 is another pleiotropic immunoregulatory cytokine (9, 10) that shares a number of biological properties with IL-4 (11–16). IL-13, like IL-4, selectively induces VCAM-1 in cultured human ECs (17, 18). IL-13 has been shown recently to stimulate tyrosine phosphorylation of the IL-4–binding subunit of the 140-kD IL-4 receptor (IL-4R) in a number of hematopoietic cell types (19), suggesting that IL-4 and IL-13 may activate a common tyrosine kinase. Two recent findings further suggest that the IL-13–binding subunit of the IL-13 receptor (IL-13R) may share at least one subunit with the IL-4R: (*a*) the IL-4 mutant protein Y124D, which inhibits IL-4–dependent reactions (20) also inhibits effects of IL-13 (21, 22); and (*b*) IL-13 competes with radiolabeled IL-4 for binding to some cells (21–24).

The IL-2 receptor (IL-2R) γ chain is a functional component of the IL-4R in lymphocytes, required for tyrosine phosphorylation of the insulin receptor substrate-1 in response to IL-4 (25) and for IL-4–induced cell proliferation (26). The (IL-2R) γ chain, recently renamed the common γ chain (γ_c chain), is also a common receptor component of many other members of the cytokine receptor superfamily including IL-7 (27–29), IL-9 (27, 30–32), and IL-15 (33, 34). It is not known if IL-13 can signal through γ_c, but some cell lines of hematopoietic origin are known to respond to IL-4 and IL-13 in the absence of γ_c (24, 35). This evidence suggests two distinct signaling pathways for IL-4, one involving γ_c, and an alternate pathway, shared with IL-13, that does not use γ_c.

Previous work by our laboratory has shown that IL-4 activates a protein tyrosine kinase that phosphorylates the IL-4R binding subunit in cultured human ECs and that activation of this protein tyrosine kinase may play a part in the signaling pathway leading to increased VCAM-1 expression in response to IL-4 (36). We now show that IL-13, like IL-4, causes the activation of a protein tyrosine kinase in ECs that phosphorylates IL-4R, that both cytokines activate JAK2 and the Stat6 transcription factor, and that these responses cannot involve γ_c, since this protein is not expressed in ECs.

Methods

Cell isolation and culture. ECs were isolated and cultured as described previously (37, 38). ECs used in these experiments were of passage levels 2 to 5 and were free of detectable $CD45^+$ leukocytes. PHA-

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1. *Abbreviations used in this paper:* EC, endothelial cell; JAK, Janus kinase; RT, reverse transcription; Stat, signal transducer and activator of transcription; VCAM-1, vascular cell adhesion molecule-1.

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stimulated PBMCs, used as a positive control for expression of the IL-4R binding subunit and γ_c, were isolated from peripheral blood obtained from adult volunteer donors using centrifugation through Lymphocyte Separation Media according to the instructions of the manufacturer (Organon Teknika, Rockville, MD). Before analysis, purified PBMCs were cultured for 48 h in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal calf serum, 60 U/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, and 5 μg/ml PHA (Sigma Immunochemicals, St. Louis, MO).

Cytokines and antibodies. Human rIL-4 (5 μg/ml) and human rIL-13 (5 μg/ml) were purchased from R&D Systems (Minneapolis, MN). PMA was purchased from Sigma. Human IFN-α (1×10^6 U/ml) was purchased from Lee Biomolecular Research Labs (San Diego, CA), human rIFN-α2b (1×10^6 U/ml), used in the Janus kinase activation studies, was purchased from Schering Corp. (Kenilworth, NJ), and human rIFN-γ (1×10^6 U/ml) was a gift from Biogen (Cambridge, MA).

The antiphosphotyrosine mouse mAb, clone 4G10, and the rabbit polyclonal antibodies to JAK1, JAK2, and TYK2, used for Western blotting and immunoprecipitation, were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The mouse anti–human IL-4 receptor mAb (M362, IgG_1), used for FACS® analysis, and the rabbit anti–human IL-4 receptor polyclonal Ab (P7), used for Western blotting, were the kind gifts of E. Thomas (Immunex Research Corporation, Seattle, WA). Other murine mAbs used for FACS® analysis were: K16/16 (irrelevant IgG_1 control, a gift of D.L. Mendrick, Brigham and Woman's Hospital, Boston, MA), 3G11 (anti–IL-2Rγ, IgM [39], a gift of J. Ritz, Dana-Farber Cancer Institute, Boston, MA), and mouse IgM controls (Sigma). Positive antibody controls used were anti–HLA-ABC (W6/32, IgG_{2a}) and anti–β_2-microglobulin (IgM, purchased from PharMingen, San Diego, CA). Antibodies used for gel shift analysis were mouse anti-Stat1α (C-111) and rabbit anti-Stat6 (S-20), both from Santa Cruz Biotechnology (Santa Cruz, CA).

FACS® analysis. FACS® analysis of indirect immunofluorescence of untreated or cytokine-treated cells was performed as described elsewhere (36). Data are presented as histograms of cell number (*y* axis) versus fluorescence intensity (log scale, *x* axis), or as corrected mean fluorescence after subtraction of nonbinding isotype-matched control mAb.

Reverse transcription (RT)-PCR analysis. Total mRNA was prepared from resting or cytokine-treated EC or from PHA-treated PBMC by lysis of the cells in guanidinium isothiocyanate followed by purification over glassMAX spin columns (GIBCO-BRL). Contaminating genomic DNA was removed by digestion with DNase I for 10 min at room temperature. DNase activity was destroyed by heating for 10 min at 65°C in the presence of 2 mM EDTA. For cDNA synthesis mRNA (2 μg) was incubated with oligo$(dT)_{12–18}$ (10 μg/ml) at 70°C for 10 min and then rapidly cooled on ice. RT was carried out in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM each dATP, dCTP, dGTP, and dTTP in the presence or absence of 200 U Superscript II reverse transcriptase (GIBCO-BRL) in a total volume of 50 μl. cDNA synthesis was performed at 43°C for 1 h and the reaction was terminated by heating at 90°C for 5 min. Nonquantitative PCR amplification was performed by adding 5 μl of the RT reaction mixture to 100 μl buffer containing 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 2 mM $MgCl_2$, 1 U *Taq* polymerase, and 400 nM oligonucleotide primers. The primers used were as follows (all 5′ to 3′, upper/lower/expected size of product): IL-2Rγ, GACCACTATGCCCACTGACTC/CCTGGAGCTGAACAACAAATG/275 bp; IL-4R, GCGAGTGGAAGATGAATGGTC/CAGGTCAGCAGCAGAGTGTCG/298 bp; glyceraldehyde-3-phosphate dehydrogenase, TGAAGGTCGGAGTCAACGGATTTGGT/CA-TGTGGGCCATGAGGTCCACCAC/983 bp). PCR conditions were: initial denaturation at 95°C, 1 min, followed by 30 cycles of: 95°C, 0.5 min, 53°C, 0.5 min, 72°C, 1.5 min, followed by a final extension of 72°C for 10 min. PCR products were separated on 1.5% agarose gels (3:1 NuSieve) containing 10 μg/ml ethidium bromide. Controls included PCR amplification without prior RT.

Immunoblot analysis. Immunoblot analysis of tyrosine-phosphorylated proteins from detergent extracts of cytokine-treated ECs has been described previously (36). Phosphotyrosine-containing immunoprecipitates were prepared as described (36) and probed for IL-4R by incubating transferred proteins with polyclonal anti–IL-4R antibody at 40 μg/ml for 2 h, 20°C, and developed using the VECTSTAIN ABC Elite Method with goat anti–rabbit IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA) at 1.5 μg/ml and LumiGLO chemiluminescent Western blotting detection reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Janus kinase activation was assessed by specific immunoprecipitation followed by Western blotting for phosphotyrosine. To immunoprecipitate JAK1, JAK2, and TYK2, 1×10^7 cells were mock-treated with saline diluent or treated with IL-4 (50 ng/ml), IL-13 (50 ng/ml), IFN-γ (1,000 U/ml), or IFN-α (500 U/ml) for 5 min. Lysates were prepared as described previously (36) and precleared by addition of 1 μl of normal rabbit serum and 25 μl each protein A–Sepharose beads and GammaBind Plus Sepharose beads (both 50% slurries (vol/vol); Pharmacia, Piscataway, NJ) for 16 h at 4°C. After removing the beads by centrifugation, the supernatants were incubated with 5 μg of the JAK2 antibody on ice for 1 h. 25 μl each of the above beads was added to each sample, which were then incubated at 4°C on a rotator for 2 h. The beads were collected by centrifugation and processed as described previously (36), except that the first supernatants were transferred to fresh tubes, 5 μg of JAK1 antibody was added to each, and immunoprecipitations were carried out, as above, for a second round. A third round of immunoprecipitations was performed on the supernatants from the JAK1 immunoprecipitation, using 5 μg of the TYK2 antibody. Immunoprecipitates were resolved by 6% SDS-PAGE, transferred to Immobilon PVDF, and assayed for Janus kinase activation by probing with the antiphosphotyrosine monoclonal antibody 4G10 and developed as described elsewhere (36). Blots were then stripped in 100 mM β-mercaptoethanol (Sigma), 2% SDS, and 62.5 mM Tris, pH 6.7, at 60°C for 30 min, washed, reblocked, and probed with 1 μg/ml of the appropriate specific anti–JAK antibody to ensure equal loading of the lanes.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Preparation of nuclear extracts has been described elsewhere (40). Nuclear proteins were extracted from $\sim 3 \times 10^6$ EC. The protease/phosphatase inhibitor cocktail contained 1 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 mM PMSF (all from Sigma), 1 μM DTT (GIBCO-BRL), 1 mM sodium fluoride, and 2 mM sodium orthovanadate (both from Sigma). Cells were enzymatically harvested using trysin/EDTA (GIBCO-BRL). The sequences of the oligonucleotides used are (5′ to 3′; complement sequence not shown): FcγRI, AGCTTGTATTTCCCAGAAAAGGGATC and nonspecific Oct1, TGTAATATGTAAAACATTTTGA. For binding reactions, 5 μg of nuclear extract was incubated with 1 μg of poly(dI-dC) (Pharmacia), and competitors were added to 100-fold molar excess in 1 μl where indicated, for a total reaction volume of 15 μl, at room temperature for 15 min. ^{32}P-Labeled probe (10,000 cpm) was then added and the incubation continued for 15 min at room temperature. Samples (10 μl) were then withdrawn and resolved on 3.5% nondenaturing polyacrylamide gel in 0.25× Tris-borate-EDTA, and the results were visualized by autoradiography. For antibody electrophoretic mobility shift assays, 1 μl of antibody was added to the reaction mix instead of competitive inhibitors.

Results and Discussion

EC expression of cytokine receptors. In lymphocytes, the γ_c chain has been shown to be a shared component of many cytokine receptors, including IL-2, IL-4, IL-7, IL-9, and IL-15 (25–35). Human ECs, under standard culture, respond to IL-4 (1–6) but not to IL-2 (41). However, it has been reported that IFN-α pretreatment can induce IL-2 responsiveness, associated with an upregulation of the IL-2Rα and IL-2Rβ chains

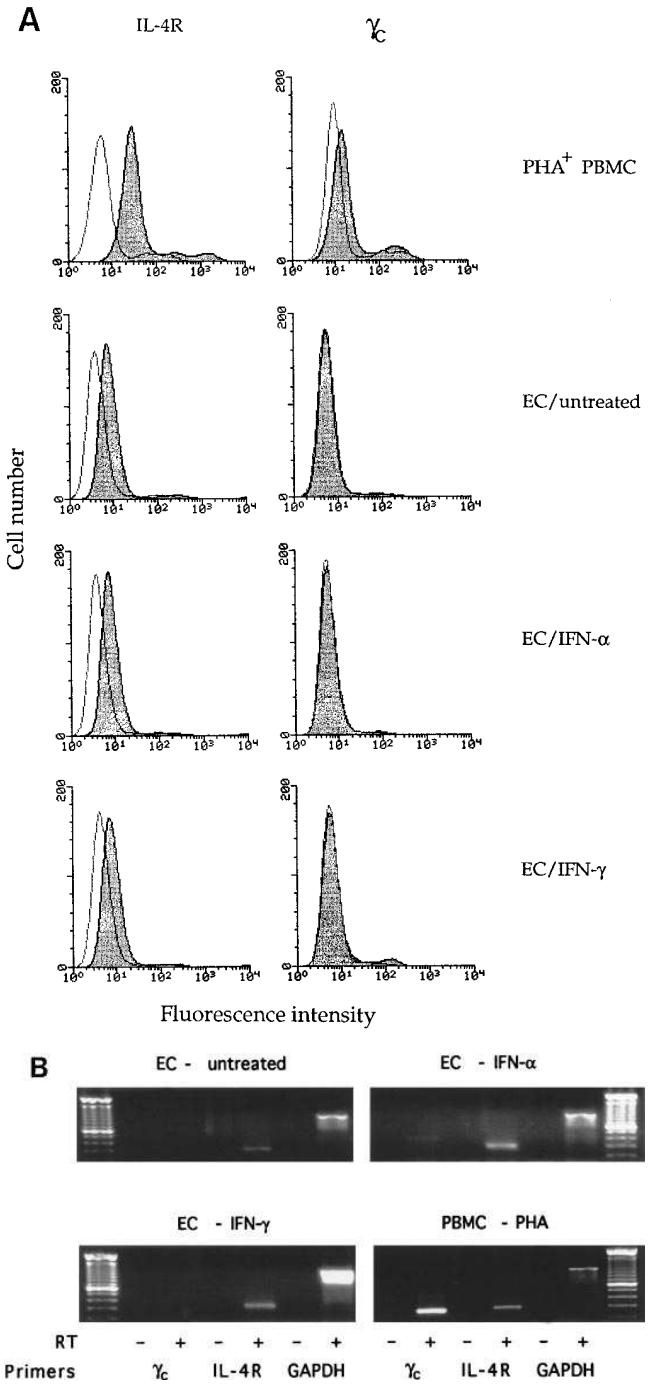


Figure 1. Expression of IL-4R and γ_c in ECs. (*A*) Cell surface expression of IL-4R or γ_c on ECs as assessed by FACS® analysis. Each panel is a histogram of either IL-4R or γ_c expression (represented by shaded curves) on ECs after 24 h of treatment with no mediator, IFN-α (1,000 U/ml), or IFN-γ (500 U/ml) as indicated. Unshaded curves represent staining with a nonbinding antibody control of the same isotype. PBMCs stimulated with PHA (5 μg/ml) for 48 h were used as a positive control. Note that IL-4R is expressed at the same level in all EC treatment groups, and that γ_c is not expressed on ECs under any of the conditions examined. Similar results were found in four independent experiments. (*B*) RT-PCR was performed on mRNA extracted from replicate cultures of ECs and PBMCs analyzed as above. Bands of the correct size for both IL-4R (298 bp) and γ_c (275 bp) are readily apparent in mRNA from activated PBMCs. Both untreated and IFN-treated ECs express IL-4R mRNA, but mRNA for γ_c is not detectable in any of the EC cultures. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) serves as a control for quality of the cDNA synthesis and is readily detectable in both PBMCs and ECs (all treatment groups). No products are observed in the absence of RT. Similar results were found in four independent experiments.

(41). The expression of the γ_c chain by human ECs has not been investigated previously. Indirect immunofluorescence, quantified by FACS® analysis, shows that human ECs express IL-4R, but that γ_c is not detectable (Fig. 1 *A*). Pretreatment of the culture with IFN-α or IFN-γ, which effectively increases HLA-A,B expression (not shown), does not augment IL-4R expression and does not induce γ_c in the same assays. PHA-activated PBMCs were used as a positive control that express both IL-4R and γ_c. The results of the FACS® analysis have been confirmed by a sensitive, nonquantitative RT-PCR analysis, shown in Fig. 1 *B*. Again, both IL-4 and γ_c transcripts can be detected in PHA-activated PBMCs, but only IL-4R is detectable in untreated or IFN-pretreated EC cultures.

The absence of γ_c in ECs raises the question of whether IL-2, which appears to act as a mitogen on IFN-α–pretreated ECs, may have an alternative γ_c-independent signaling pathway in this cell type. In preliminary experiments, we have been able to detect the expression of IL-2Rα and β on ECs in some cultures, but we have not found consistent expression or a consistent increase in expression in response to IFN-α or IFN-γ. It is also possible that IL-2–induced responses require the mediation of an additional contaminating cell type (e.g., a T cell or monocyte) that may be more abundant in some endothelial culture systems than others (42).

Tyrosine phosphorylation of the IL-4 receptor and activation of JAK. IL-4 specifically induces tyrosine phosphorylation of its receptor and other intracellular substrates in lymphoid and hematopoietic cell lines of both murine and human origin (43–45). IL-4 signaling in many of these cells has been shown to involve tyrosine phosphorylation and/or receptor association of a number of downstream mediators including the Janus family tyrosine kinases, JAK1 and JAK3 (30, 46, 47) phosphatidyl inositol 3-kinase (43, 48), or insulin receptor substrate-1 (48, 49). These events appear to involve signaling through the γ_c subunit that is also expressed in these cells. We have shown previously that IL-4 induces activation of a protein tyrosine kinase that phosphorylates the IL-4R in ECs and that activation of this tyrosine kinase may be necessary for IL-4–induced VCAM-1 expression (36). Examination of IL-13–treated crude cell lysates by Western blot analysis with an antiphosphotyrosine mAb shows that IL-13 also induces the tyrosine phosphorylation of a single species of 145,000 M_r (Fig. 2). Immunoprecipitation of lysates from IL-13–treated ECs with an antiphosphotyrosine monoclonal antibody and Western blotting with an anti–IL-4R polyclonal antibody showed the tyrosine-phosphorylated species induced by IL-13 to be the IL-4R binding subunit (Fig. 2). Comparison of the kinetics of the IL-13–induced tyrosine phosphorylation of the IL-4R with those of the IL-4–induced response (Fig. 3) shows that the IL-13–induced tyrosine phosphorylation of this protein may be slower (onset at 10 min, peak at 30–60 min) than that induced by IL-4 (onset by 1 min, peak at 5–30 min). A slower time course for IL-13–mediated tyrosine phosphorylation has been reported in the human premyeloid erythroleukemic cell line

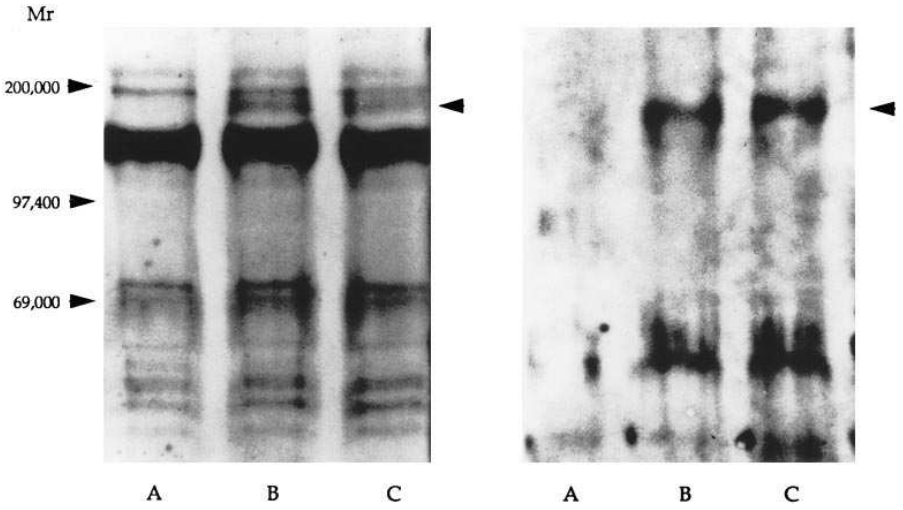
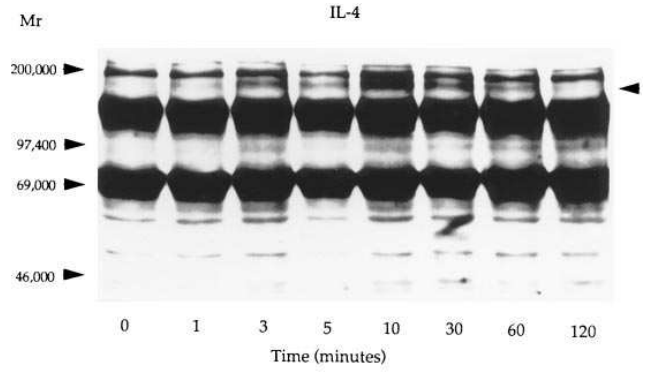


Figure 2. Identification of tyrosine-phosphorylated proteins in IL-4– or IL-13–treated cultures. Identification of tyrosine phosphorylated proteins by immunoprecipitation, followed by Western blotting for IL-4R. EC cultures were untreated (*A*), treated with 30 ng/ml IL-4 (*B*), or treated with 100 ng/ml IL-13 (*C*) for 15 min before immunoprecipitation. The three left lanes are whole cell lysates from the same cells before immunoprecipitation, probed with antiphosphotyrosine mAb. The three right lanes were immunoprecipitated with antiphosphotyrosine mAb and probed with polyclonal IL-4R antibody as described in Methods. The arrowheads indicate the position of the induced bands. Note that both IL-4 and IL-13 selectively increase tyrosine phosphorylation of IL-4R. One of three independent experiments with similar results.



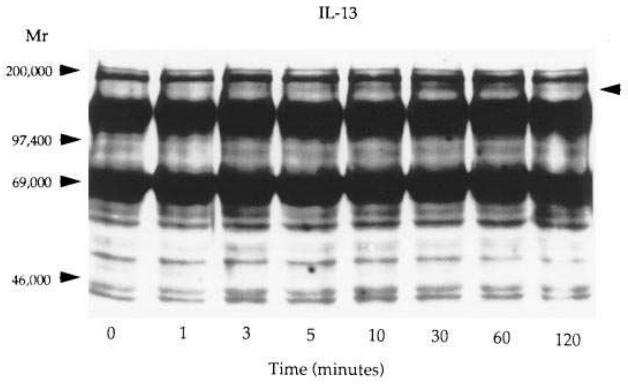


Figure 3. Time course of protein tyrosine phosphorylation, assessed by Western blotting, in IL-4– or IL-13–treated cultures. Treatments were with 20 ng/ml IL-4 or 5 ng/ml IL-13. The arrowhead indicates the position of the 145,000 M_r band. Note that the onset of tyrosine phosphorylation induced by IL-13 is slower than that induced by IL-4. One of two experiments with similar results.

TF-1, which responds to either IL-4 or IL-13 with transient proliferation (21, 50).

The identity of the tyrosine kinase that acts on the IL-4 receptor is not known. In many cells, cytokine receptors are phosphorylated by Janus family kinases. In hematopoietic cells that express the common γ chain, IL-4 is thought to activate JAK1 and JAK3 (51–53). It has been reported that in colon carcinoma cells, which lack the γ_c chain, IL-4 and IL-13 activate JAK2 as well (54, 55). As shown in Fig. 4, we find evidence for IL-4 and IL-13 activation of JAK2 in ECs that is more prominent than that induced by IFN-γ. Resting EC do not express JAK3 (our unpublished observations) and, in 10 independent experiments, we did not see evidence for activation of JAK1 or TYK2 under the same conditions, although these kinases could by activated by IFN-γ and/or IFN-α (data not shown).

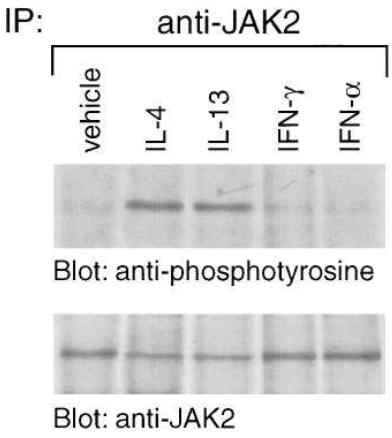


Figure 4. Tyrosine phosphorylation of JAK2 in response to IL-4 and IL-13 treatment. EC were mock-treated with saline diluent, or were treated with 50 ng/ml IL-4, 50 ng/ml IL-13, 1,000 U/ml IFN-γ, or 500 U/ml IFN-α for 5 min. Lysates from the equivalent of 1×10^7 cells were then immunoprecipitated with an antibody recognizing JAK2 and Western blotted using the antiphosphotyrosine mAb 4G10, as described in Methods. The blot was stripped and reprobed with the immunoprecipitating antibody to show equivalent loading (*bottom*). Similar results were found in 10 independent experiments.

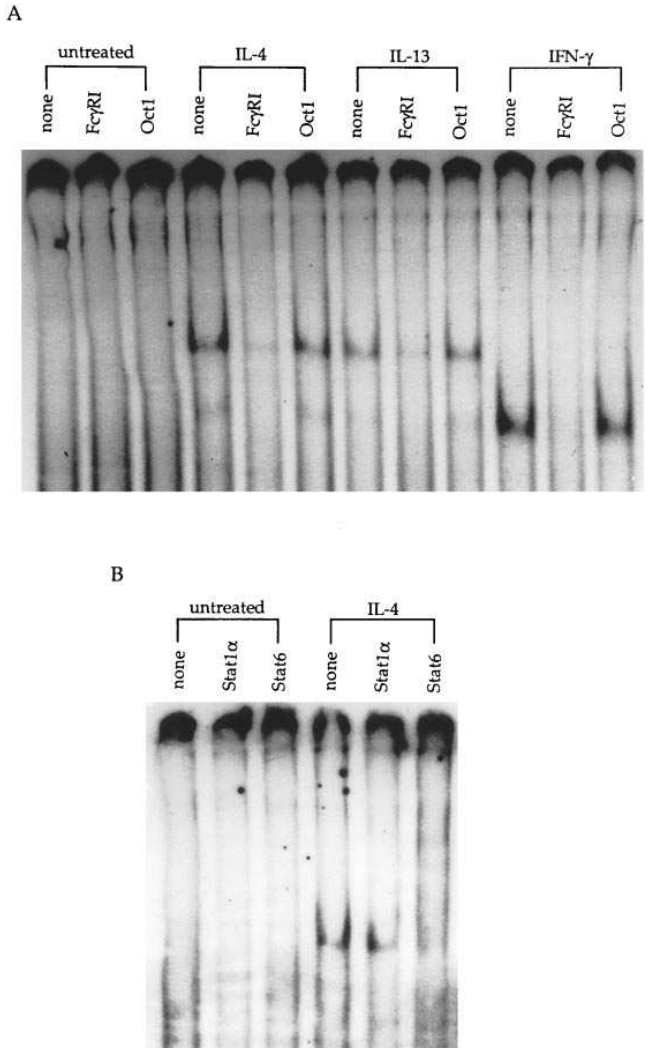


Figure 5. Activation of Stat6 by IL-4 and IL-13 in EC. Treatments were with 50 ng/ml IL-4 or IL-13, or 1,000 U/ml IFN-γ, for 15 min. (*A*) IL-4 and IL-13 induce complexes of identical mobility in EC, which are different in mobility from complexes induced by IFN-γ. The Oct1 DNA was used as nonspecific competitor DNA. Similar results were found in three independent experiments. (*B*) Binding of the IL-4–induced nuclear factor is blocked by a Stat6 antibody, but not a Stat1α antibody. Similar results were found in three independent experiments.

Activation of the Stat6 transcription factor. Many activities of IL-4 and other cytokines are now known to be mediated by the activation of Stat transcription factors (56). In hematopoietic cells, IL-4 specifically activates the Stat6 transcription factor. Interestingly, Stat6 was originally identified by cloning from a human umbilical vein EC cDNA library (57), suggesting that it could mediate IL-4 responses in this cell type as well. Recently, it has been reported that IL-4 and IL-13 activate the same DNA-binding protein in transfected COS cells that lacked the γ_c chain (24). We find that in ECs both IL-4 and IL-13 activate a Stat protein that forms complexes which migrate more slowly that those activated by IFN-γ (Fig. 5 *A*). Antibody to Stat6 blocked the complexes activated by IL-4; whereas antibody to Stat1α, known to be activated by IFN-γ (reference 56 and data not shown), did not block these complexes (Fig. 5 *B*). We conclude that both IL-4 and IL-13 activate Stat6 in human ECs.

We do not yet know whether Stat6 is involved in VCAM-1 expression. It is likely that both IL-4 and IL-13 activate transcription of this protein since VCAM-1 mRNA is not present in untreated cells. However, at later times, the primary effect of these cytokines may be largely posttranscriptional (6). The coupling of Stat6 activation to VCAM-1 transcription, if it exists, is likely to be indirect since the protein synthesis inhibitor cycloheximide can block accumulation of VCAM-1 mRNA in response to IL-13 (18). Studies to address these questions are in progress.

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