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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*

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Functional receptors for interleukin (IL)-4 and IL-13 on endothelial cells consist of the 130-kDa IL-4 receptor α-chain (IL-4Rα) and a 65–75-kDa IL-13 binding subunit that are expressed in a ratio of about 1:3, respectively. The restricted number of IL-4Rα limits subunit heterodimerization and in turn receptor-mediated signaling. We report here, the effects of tumor necrosis factor α (TNF-α) on the expression of the receptor subunits for IL-4 and IL-13. By flow cytofluorometry and receptor-binding analysis of iodinated IL-4 and IL-13, stimulation with TNF-α-induced a 2–3-fold increase of the IL-4Rα expression. The up-regulation was also confirmed at the transcriptional level by reverse transcription-polymerase chain reaction. Radioligand cross-linking experiments revealed no change in the subunit composition of the TNF-α-induced receptor complex. Nevertheless, TNF-α stimulation led to increased activation of the IL-4-specific signal transducers and activators of transcription protein (Stat6) by IL-4 and IL-13. Thus, TNF-α corrects the subunit imbalance of the endothelial IL-4·IL-13 receptor complex thereby increasing receptor heterodimerization and in turn the signaling capability by IL-4 and IL-13.

Vascular endothelial cells participate actively in a wide variety of pathophysiological processes, including inflammation and immunity (1). Inflammatory cells secrete cytokines that bind to endothelial cells and result in changes of cell surface molecule expression. The T_H1-type proinflammatory cytokines TNF-α[1] and IL-1, as well as bacterial endotoxin, induce the expression of endothelial cell adhesion molecules like E-selectin (2) intercellular cell adhesion molecule type-1 (ICAM-1) and vascular cell adhesion molecule type-1 (VCAM-1) that are the basis for the coordinated extravasation of the different types of leukocytes (for review see Ref. 3).

Through the sharing of receptor components, T_H2-type cytokines IL-4 and IL-13 exert a similar spectrum of responses on different leukocytic cells (4). Both interact with human umbilical vein endothelial cells (HUVEC) and induce, like TNF-α and IL-1, the expression of VCAM-1 and in turn the adhesion of very late antigen-4-expressing leukocytes, including eosinophils (5, 6), as well as their selective transmigration (7, 8). These studies indicate that IL-4 and IL-13 play an important regulatory role during allergic inflammation.

Recently, we characterized the receptors for IL-4 and IL-13 on human endothelial cells as a heterodimeric complex lacking expression of the common γ-chain (γ_c) (8), which is otherwise shared by IL-2R, IL-4R, IL-7R, IL-9R, and IL-15R (9, 10). The subunit structure of the functional receptors for IL-4 and IL-13 on endothelial cells consists of the 130-kDa IL-4R α-chain (IL-4Rα) and a 65–75-kDa IL-13-binding protein herein called the IL-13R α-chain (IL-13Rα). A mouse and recently a human IL-13R have been cloned and characterized as new members of the hematopoietin receptor family (11, 12). IL-4 signals through receptor association and activation of the Janus family of tyrosine kinases (13) Jak1 and Jak3 in T cells, natural killer cells, and myeloid cells (14, 15). Hereby Jak1 associates with IL-4Rα (16) and Jak3 with γ_c (17, 18). Mutations in γ_c preventing association with Jak3 result in human X-linked (severe) combined immunodeficiency and X-linked combined immunodeficiency (17). In human colon carcinoma cells, expressing a heterodimeric IL-4·IL-13R complex lacking γ_c; however, IL-4 and IL-13 activate Jak1, Jak2, and Tyk2 but not Jak3 (19, 20), and in myeloid cells (21) IL-13 has been reported to activate Jak1 as well. Activated Jak kinases mediate cellular responses by phosphorylating members of the STAT family (Signal Transducers and Activators of Transcription), a class of latent cytoplasmatic transcription factors, which translocate into the nucleus and transcriptionally activate target genes (22). IL-4 has been reported to activate Stat6, formerly designated IL-4 Stat (23), and IL-13 activates in human colon carcinoma cells Stat6 as well (19, 20).

Here we describe the effects of proinflammatory cytokines,

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[1] The abbreviations used are: TNF-α, tumor necrosis factor α; IL, interleukin; ICAM-1, intercellular cell adhesion molecule type-1; VCAM-1, vascular cell adhesion molecule type-1; HUVEC, human umbilical vein endothelial cells; Jak, Janus family tyrosine kinase; STAT, signal transducers and activators of transcription; IL-4Rα and IL-13Rα, interleukin 4 and interleukin 13 receptor α-chain; γ_c, common γ-chain; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MFI, mean fluorescence intensity; EMSA, electrophoretic mobility shift assays; PBS, phosphate-buffered saline; Me_2SO, dimethyl sulfoxide; mAb, monoclonal antibody; bp, base pair.

TNF-α and IL-1, on the expression of the receptor subunits for IL-4 and IL-13 on HUVECs in culture. Stimulation with TNF-α led to a 2–3-fold increase of the IL-4Rα expression, without affecting the number of the IL-13Rα subunits. Concerning the numeric imbalance of the receptor subunits determined in resting HUVECs (8), endothelial activation induced an impressive shift toward a 1:1 ratio of both receptor subunits. The resulting subunit equilibration revealed an increased binding of both IL-4 and IL-13 to the IL-4·IL-13R complex. Furthermore, pretreatment of HUVECs with TNF-α resulted in increased Stat6 activation demonstrating improved signaling capability of the induced IL-4·IL-13R complex.

MATERIALS AND METHODS

Cytokines and Reagents—The Chinese hamster ovary-derived human IL-4 together with the human IL4R α-chain-specific mAb S697 were a gift of Dr. J. Banchereau (Schering Plough, Dardilly, France). The S697 antibody is of the mouse IgG1 class and reacts with the extracellular domain of the human IL-4Rα (Zurawski *et al.* (35)). The mAb 1299-27-1 directed against VCAM-1 is of the mouse IgG_{2a} class and was purchased from BMA Biomedicals AG, Augst, Switzerland. The mAb M2-specific for the flag octapeptide was from Eastman Kodak Co. Human IL-4 has been biotinylated with biotin-succinimidyl ester (Amersham, Buckinghamshire, UK). Briefly, 500 μg of IL4 (1 mg/ml) in 0.1 M $NaHCO_3$ (pH 8.5) was added to 125 μg of biotin-succinimidyl ester (10 mg/ml in dimethyl sulfoxide (Me_2SO)) and incubated for 4 h at room temperature. Labeling reaction was stopped by adding 20 μl of 1 M NH_4Cl and incubating for 10 min at room temperature. The reaction mixture was then extensively dialyzed against phosphate-buffered saline (PBS) to remove free biotin and side products of the reaction. Human IL-1 was generously supplied by Dr. P. T. Lomedico (Hoffman-La Roche Ltd., Basel, Switzerland), human TNF-α by Dr. Z. Nagy (Preclinical Research, Sandoz Ltd., Basel, Switzerland), TNF receptor (TNFR)-p55-specific TNF-α mutant Trp^{32}–Thr^{86}TNF-α and TNFR-p75-specific TNF-α mutant Asn^{143}–Arg^{145}TNF-α by Dr. H. Loetscher (Pharmaceutical Research, F. Hoffman-La Roche Ltd., Basel, Switzerland). Lipopolysaccharide (from *Salmonella minnesota*) were purchased from Sigma. Herbimycin A, genistein, and staurosporine (Calbiochem) were diluted in Me_2SO and used at indicated concentrations with a final concentration of Me_2SO 1:1000 v/v.

Cell Cultures—HUVECs were isolated from human umbilical cord veins as described previously (24). The cells were seeded on purified human fibronectin (Winiger AG, CH-5610 Wohlen, Switzerland) and propagated in Medium 199 enriched with sodium heparin (90 μg/ml; Novo Industries, Copenhagen, Denmark), endothelial cell growth supplement (15 μg/ml; Collaborative Research, Inc., St. Waltham, MA) in the presence of 20% pooled human serum. Final monolayers were used in their second to fourth passage exhibiting cytoplasmic factor VIII von Willebrand, which was tested by indirect immunofluorescence with the mAb KG7/30 (BMA Biomedicals AG, Augst, Switzerland). THP1 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 nutrient solution (Life Technologies, Inc., Basel, Switzerland) containing 10% fetal calf serum.

Production of FLAG-labeled Human IL-13—FLAG-labeled human IL-13 containing the N-terminal octapeptide *N*-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-C was produced by subcloning the mature peptide coding region for human IL-13 (25) via PCR into the pFLAG 1 vector (Kodak) and by transfection into the AB1899 strain of *Escherichia coli*. Bacterial cultures were harvested, clarified, and supernatants adjusted to PBS and recirculated overnight at 4 °C through a 5-ml bed volume of M2 resin, eluted with 100 mM glycine-HCl (pH 3.0), neutralized with 1:50 (v/v) 2 M Tris-HCl (pH 8.0), and dialyzed against PBS. Protein was quantitated by densitometer scanning (Molecular Dynamics Corp., Sunnyvale, CA) of 10% SDS-polyacrylamide gels stained with Coomassie Brilliant Blue (R030–250) using hen egg lysozyme as a standard with verification by amino acid analysis.

Flow Cytofluorometric Analysis—HUVECs were grown to confluence in 6-well plates (Becton Dickinson, San José, CA). Reagents were added directly to the medium at indicated concentrations and for given incubation times. Flow cytometric detection of IL4Rα and VCAM-1 expression was essentially performed as described previously (8). For analysis of IL4 and IL13 binding to cell surface, samples were incubated with IL-4-biotin or IL-13-FLAG (100 nM) for 2 h at 4 °C followed by several wash steps. IL-13-FLAG-labeled cells were additionally incubated with mAb M2 anti-flag (10 μg/ml) for 45 min at 4 °C. Cells were further incubated for 45 min at 4 °C with a 1:30 dilution of goat anti-mouse IgG-phycoerythrin R or a 1:10 dilution of streptavidin/phycoerythrin R (Sigma). Flow cytofluorometry was carried out using a 488-nm argon laser FACScan (Becton Dickinson, San Jose, CA). 10,000 cells/sample were measured, and fluorescence intensity was determined on a linear scale by the LYSIS II software (Becton Dickinson). Cells were gated using forward *versus* side scatter to exclude dead cells and debris. Results are expressed as mean fluorescence intensities (MFI) after subtraction of unspecific binding of a irrelevant isotype-matched control antibody or as percentage immunopositive stained cells.

Iodination of IL-4 and IL-13—Iodination of the cytokines was performed as described recently (8). The specific activities of ^{125}I-labeled IL-4 and ^{125}I-labeled IL-13 were 60–70 and 90–100 μCi/mg, respectively. Iodination did not lead to a detectable loss of the biological activity of the cytokines.

Affinity Cross-linking—Confluent HUVEC monolayers were briefly trypsinized. The cells were washed three times with cold PBS, counted at the last washing step, and resuspended in minimum Eagle's α medium containing 1% bovine serum albumin. Aliquots of 200 μl containing 4×10^6 HUVECs were incubated on ice for 1 h with 0.5 nM ^{125}I-labeled IL-4 and 3 nM ^{125}I-labeled IL-13. For competition, an excess of 1 μM of unlabeled ligand was added 20 min prior to the iodinated cytokines. The affinity cross-linking procedure was performed as described recently (8), and the samples were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions using 3–10% (w/v) polyacrylamide gels. Autoradiography was performed at −70 °C exposing x-ray Hyperfilms (Amersham Int.) for 2–3 weeks.

Binding of ^{125}I-Labeled IL-4 and ^{125}I-Labeled IL-13 to Endothelial Cells—Confluent HUVEC monolayers were briefly trypsinized, washed three times with cold PBS, and resuspended in minimum Eagle's α medium containing 1% bovine serum albumin. The binding studies were performed as described previously (8). Nonspecific binding was determined by incubating the same number of cells with a 1000-fold excess of unlabeled IL-4 or IL-13 at 4 °C for 30 min. Binding data were analyzed with the computerized weighted least-square curve fitting software described by Munson and Rodbard (26).

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)—Total cellular RNA was isolated by the method of Chomczynski *et al.* (27) and quantitated at 260 nm. 5 μg of total cellular RNA were reverse-transcribed in a final reaction volume of 30 μl as recently described (8). 1 μl of this reaction was then amplified by PCR. The specific primer pairs for the human IL4Rα and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were custom synthesized and quality checked by capillary electrophoresis by MWG-Biotech (85560 Ebersberg, Germany). The human GAPDH primers spanning a 480-bp fragment and the human IL4-receptor α-chain primers spanning a fragment of 571 bp were as described (8, 28). The presumptive human IL-13R transcript was amplified using primers corresponding to nucleotides 25–43 of Hs074281 and nucleotides 302–323 of Hs334240 spanning a fragment of 692 bp. PCR reactions were carried out in a final volume of 25 μl in 500-μl microtubes (Perkin-Elmer), and each sample was overlaid with 25 μl of paraffin oil. The mixtures contained 1 μl of RT reaction in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.6 mM $MgCl_2$ (IL-4Rα and IL-13Rα), 0.8 mM $MgCl_2$ (GAPDH), 0.01% gelatin, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.8 μM of each primer, and 0.5 units of Super *Taq* polymerase (P. H. Stehelin & Cie AG, Basel, Switzerland). Human GAPDH was amplified using 35 cycles at 94 °C (for 1 min), at 62 °C (for 1 min), and at 72 °C (for 1 min), IL-4Rα using 35 cycles at 94 °C (for 30 s) and at 53 °C (for 30 s), and IL-13R using 35 cycles at 94 °C (for 1 min), at 50 °C (for 2 min), and at 72 °C (for 2 min). The identity of the RT-PCR products of IL-4Rα and the human homolog of the mouse IL-13R (11) was confirmed by sequencing. To control for genomic DNA amplification, PCR analyses were done without reversed transcription. In all cases no products of corresponding size could be amplified. Twenty microliters of each RT-PCR reaction were run on a 1.5% agarose gel containing 0.2 μg/ml ethidium bromide in 1 × TAE buffer. pBR322 DNA completely digested by *Alu*I (MBI Fermentas, Vilnius, Lithuania) was used as DNA molecular weight marker. The gels were photographed under UV illumination.

Electrophorectic Mobility Shift Assays (EMSA)—HUVECs were grown to confluence and either left untreated or stimulated with 1 ng/ml TNF-α for 18 h before activation with the indicated concentrations of IL-4 or IL-13 for 15 min. Nuclear extracts were prepared with a modified Nonidet P-40 method as follows. Cells were lysed in low salt buffer (20 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM $NaVO_4$, 1 mM EDTA, 1 mM EGTA, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) for 10 min on ice. Lysates were centrifuged in a microcentrifuge at 13,000 × *g* for 1 min at 4 °C. Supernatants were

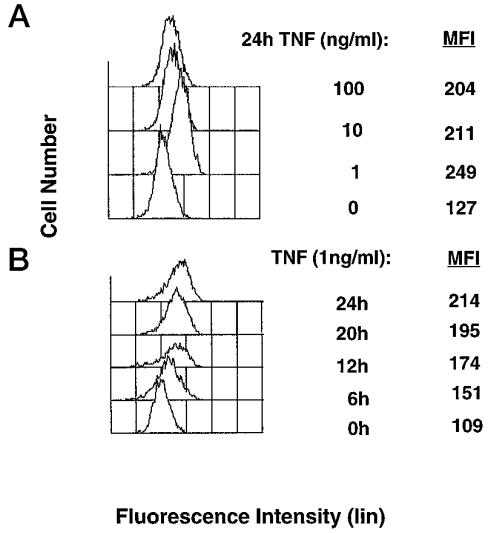

FIG. 1. **Dose dependence and kinetics of IL-4Rα induction by TNF-α.** HUVEC monolayers were incubated in medium with increasing concentrations of TNF-α for 24 h (*A*) or with TNF-α at 1 ng/ml for the indicated periods (*B*). Flow cytofluorometry for cell surface expression of IL-4Rα was performed using the specific mAb S697 as primary antibody. Results are from one representative experiment using the same batch of endothelial cells in *A* and *B*, respectively. Data are expressed as corrected MFI.

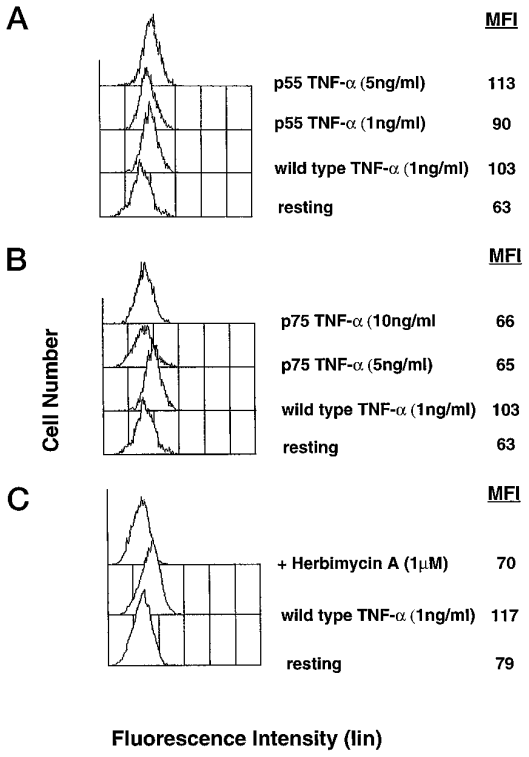

FIG. 2. **Up-regulation of IL-4Rα is mediated through the p55 TNFR and involves the activation of a protein tyrosine kinase.** HUVEC were stimulated in culture with wild-type TNF-α or Trp^{32}–Thr^{86}TNF-α (p55 TNF-α) (*A*) or with Asn^{143}–Arg^{145}TNF-α (p75 TNF-α) (*B*) at the indicated concentrations for 24 h. *C*, HUVEC were incubated for 2 h with either Me_2SO (1:1,000 v/v) (resting) or herbimycin A before stimulation with TNF-α for 24 h. Flow cytofluorometry for cell surface expression of IL-4Rα was performed using the specific mAb S697 as primary antibody. Values represent corrected MFI from one representative experiment.

frozen on dry ice and stored at −70 °C as cytoplasmic extracts. The pellets were resuspended in high salt buffer (420 mM NaCl, 20 mg/ml Hepes (pH 7.9), 10 mM KCl, 0.1 mM $NaVO_4$, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and incubated on ice for 30 min. These nuclear extracts were cleared by centrifugation at 13,000 × *g* in a microcentrifuge for 5 min. The supernatants were frozen on dry ice and stored at −70 °C until used. 2 μl of nuclear extracts were used for EMSA analysis. EMSA was done as described previously (29) using an oligonucleotide probe derived from the Cε gene promotor (30, 31) with the core sequence TTCCCAAGAA. For supershifts, extracts were mixed with 1 μl of a 1:10 dilution of specific antisera against Stat1 (32), Stat2 (33), Stat3, Stat4 (34), Stat5, and Stat6. Stat5 antiserum was raised in rabbits against amino acids 687–794 of ovine Stat5. Polyclonal rabbit antiserum against Stat6 was raised in rabbits against amino acids 633–837 of mouse Stat6.

RESULTS

TNF-α Up-regulates the Expression of IL-4Rα on HUVEC Monolayers—HUVECs express a functional IL-4·IL-13R complex that consists of the IL-4Rα and a 65–75-kDa IL-13-binding protein, the IL-13Rα, and lacks the common γ_c (8). By flow cytofluorometry we studied the expression of the IL-4Rα with the non-blocking mAb S697 (35). Untreated HUVECs in culture constitutively expressed the IL-4Rα, although the amount varied considerably among the different cell batches (MFI, 171 ± 15, mean ± S.E. of 20 experiments). After activation with TNF-α at a concentration of 1 ng/ml for 24 h, we observed significant increase of the IL-4Rα expression (MFI, 251 ± 17, mean ± S.E. of 20 experiments; $p < 0.001$). Similar up-regulation was obtained with 10 ng/ml IL-1 or lipopolysaccharide at a concentration of 1 μg/ml (data not shown). Increased expression of IL-4Rα was detectable at a TNF-α concentration of 0.1 ng/ml and was optimal at 1 ng/ml. Higher concentrations were less efficient, probably due to TNF-α-mediated toxicity (Fig. 1*A*). After 6 h the IL-4Rα expression was significantly enhanced and linearly increased up to 24 h (Fig. 1*B*). Prolonged incubation led to decreased IL-4Rα expression, probably due to desensitization (data not shown). Of importance, TNF-α stimulation did not induce γ_c (data not shown). Moreover, the IL-4Rα was not inducible in synovial and dermal fibroblasts as well as in the human mast cell line 1 expressing the γ_c-containing heterotrimeric form of the IL-4·IL-13R (36) (data not shown). This is indicative for a specific regulatory mechanism in endothelial cells.

Endothelial cells are known to express both the p55 and p75 TNFR (37). A specific role of the p55 TNF receptor was reported for the induction of ICAM-1, E-selectin, VCAM-1, and CD44. However, the expression of the α_2/β_1 integrin was shown to be mediated through both the p55 and p75 TNFR (37, 38). To investigate which TNFR controls up-regulation of the IL-4Rα, HUVECs were stimulated for 24 h either with wild-type TNF-α (1 ng/ml) or with receptor type-specific agonists (39), binding exclusively to p55 or p75 TNFRs. The p55 TNFR selective mutant of TNF-α (p55 TNF-α) binds with the same affinity as wild-type TNF-α (39). At a concentration of 5 ng/ml, it fully up-regulates the expression of IL-4Rα in our experiments (Fig. 2*A*). Conversely, the p75 TNFR selective mutant (p75 TNF-α) (Fig. 2*B*) with a 5–10-fold lower affinity for p75 TNFR did not exhibit any inducing effect at concentrations of 5 or 10 ng/ml (Fig. 2*B*), and even at 50 ng/ml (data not shown).

TNF-α is known to signal through the activation of various kinases, like cAMP-dependent protein kinase (40), protein kinase C (41), and a $pp60^{src}$-like tyrosine kinase (42). To investigate the TNF-α-dependent signaling pathway leading to enhanced expression of IL-4Rα, HUVECs were pretreated with kinase inhibitors either specific for tyrosine kinases as herbimycin A and genistein (data not shown) or for protein kinase C as staurosporine (data not shown) for 2 h at 37 °C and coincubated with TNF-α (1 ng/ml) for an additional period of 24 h. Only treatment with herbimycin A, a specific $pp60^{src}$ tyrosine

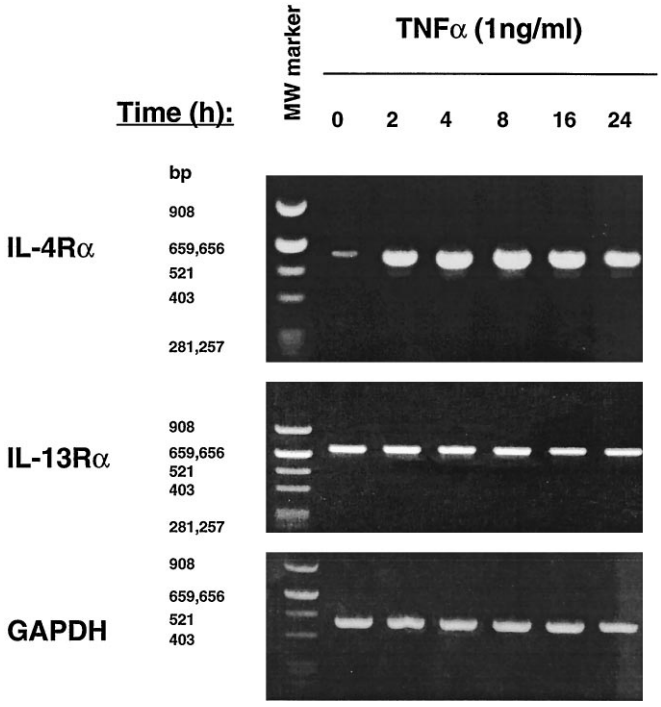

FIG. 3. **Reverse transcriptase-PCR analysis of IL-4Rα mRNA in presence of TNF-α.** Total cDNA was synthesized from 5 μg of total cellular RNA harvested from HUVECs stimulated in culture with TNF-α for the times indicated (*h*). PCR amplification was performed as described under "Material and Methods." Specific primer pairs were used to detect IL-4Rα mRNA, the presumptive IL-13Rα mRNA and GAPDH mRNA expression for equal amplification. PCR products were analyzed by electrophoresis through ethidium bromide-stained agarose gels. The gels were photographed under UV illumination. The results are from one representative experiment using the same batch of endothelial cells.

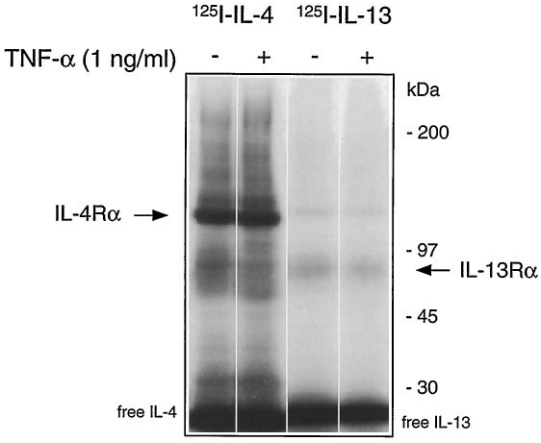

FIG. 4. **Radioligand affinity cross-linking of IL-4 and IL-13 to HUVECs.** Confluent HUVECs either resting (−) or activated for 24 h with 1 ng/ml TNF-a (+) were detached and labeled with 0.5 nmol/liter ^{125}I-labeled IL-4 (*lanes 1* and *2*) or 3 nmol/liter ^{125}I-labeled IL-13 (*lanes 3* and *4*) before cross-linking was performed using 2.5 mmol/liter disuccinimidyl suberate. The lysates were analyzed under reducing conditions on a gradient (3–10%) of SDS-polyacrylamide gel electrophoresis and exposed to x-ray films for 3 weeks at −70 °C. Net molecular masses of the receptors were calculated by subtracting 19 kDa for bound IL-4 or 15 kDa for bound IL-13.

kinase inhibitor, at a concentration of 1 μM resulted in complete inhibition of the IL-4Rα up-regulation (Fig. 2*C*).

TNF-α Stimulates the Expression of IL-4Rα mRNA—On mouse sarcoma cells, TNF-α has been reported to increase IL-4 receptor-specific mRNA levels with maximal transcript up-regulation after 4 h of TNF-α treatment, although the requirement for protein synthesis could not be clearly determined (43).

By RT-PCR analysis we studied the expression of IL-4Rα-specific mRNA of resting HUVECs and HUVECs previously stimulated with TNF-α for various periods (Fig. 1*B*). With appropriate primers (see "Materials and Methods"), which amplify a 571-bp fragment encoding the IL-4Rα, specific transcripts were detectable in resting HUVEC and were clearly increased after TNF-α stimulation. However, a 692-bp transcript with close homology to the mouse IL-13R (11) was constitutively expressed in resting HUVEC and was not increased after TNF-α stimulation (Fig. 3). In addition, we were unable to detect any message for γ_c (data not shown), as previously reported for resting HUVECs (8).

TNF-α Does Not Change the Subunit Composition of the IL-4·IL-13R Complex—To investigate whether the subunit structure of the receptor complex has changed in response to TNF-α treatment, iodinated IL-4 and IL-13 were cross-linked by the succinimidyl suberate method to their binding sites on the surface of endothelial cells (Fig. 4). Autoradiographies of SDS-polyacrylamide gel electrophoresis from resting and TNF-treated HUVECs revealed for both cytokines exactly the same pattern of interacting subunits as recently reported (8). In detail, the majority of the iodinated IL-4 was cross-linked to the 130-kDa IL-4Rα, whereas a minor band was detected at 65–75 kDa. However, iodinated IL-13 bound predominantly to a 65–75-kDa protein, and only a trace amount of radioactivity was detected at the 130-kDa level. Total displacement of the iodinated IL-4 and IL-13 by an excess of the respective unlabeled cytokine (1 μmol/L) indicated the specificity of the interaction (data not shown). From these experiments we conclude that stimulation of endothelial cells does not modify the subunit composition of the IL-4·IL-13R complex.

TNF-α Increases the Binding of Biotinylated IL-4 and Flagged-IL-13 to HUVECs—Concerning the heterodimeric structure of the IL-4·IL-13R complex on endothelial cells, we studied the effect of TNF-α stimulation on binding of both IL-4 and IL-13. Flow cytofluorometric analysis was performed with biotinylated IL-4 and flagged IL-13. Resting HUVECs showed detectable binding of biotinylated IL-4 and flagged IL-13 both at 100 nM and after activation with TNF-α for 24 h, the binding of both ligands was 2–3-fold increased (Fig. 5*A*). In all experiments, binding of biotinylated IL-4 or FLAG-labeled IL-13 could be effectively competed by addition of excess unlabeled IL-4 or IL-13 (data not shown).

Receptor-binding Analysis of Radiolabeled IL-4 and IL-13—In previous experiments we studied the binding of iodinated IL-4 and IL-13 to resting HUVECs and analyzed binding of iodinated IL-4 and IL-13 to resting HUVECs using a one-site binding model (8). These calculations revealed with ~200 IL-4 receptors/cell and ~700 IL-13 receptors/cell a considerable stoichiometric imbalance of the receptor subunits. Here we have compared the binding characteristics of resting and TNF-activated HUVECs and analyzed the binding data by the weighted least-square curve fitting method regarding one-class or two-class binding site models (26). Binding of IL-4 in resting and TNF-α-activated HUVECs fitted with high confidence to the one-class binding site model ($p < 0.02$). In resting HUVECs, IL-4 bound to 228 ± 82 receptors per cell expressing a K_d of 33.0 ± 13.0 pM. After TNF activation there was 2.7-fold increase of the number of receptors/cell, whereas the K_d value remained unchanged (Fig. 5*B*). Binding of IL-13 to resting HUVECs fitted the two-class rather than the one-class binding site model ($p < 0.02$) that suggests 142 ± 24 receptors/cell with a $K_{d\,1}$ of 7.4 ± 1.7 pM. According to the two-class binding site theory a second receptor population is predicted with a $K_{d\,2}$ of 4.9 ± 3.0 nM and 575 ± 144 sites/cell (Fig. 5*B*). In contrast, the

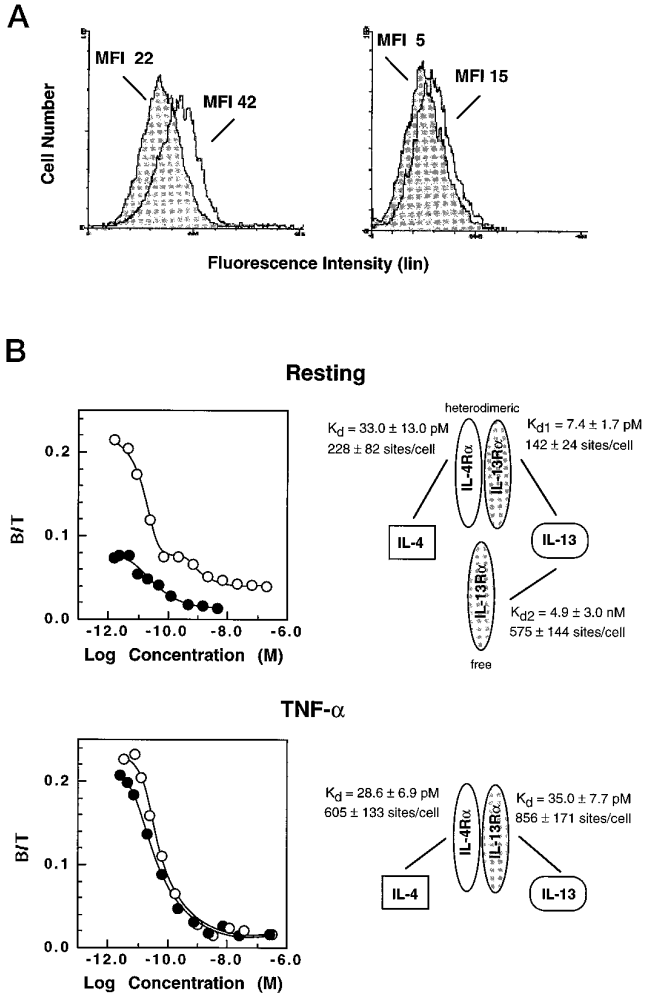

FIG. 5. **Binding of IL-4 and IL-13 to resting and TNF-α-activated HUVECs.** *A*, flow cytofluorometry for cell surface binding of IL-4-biotin (*left*) or IL-13-FLAG (*right*). HUVEC monolayers were stimulated with TNF-α (1 ng/ml) or medium alone (*Resting; gray filled histograms*) for 24 h. Data are expressed as corrected MFI from one representative experiment. *B*, binding of radiolabeled IL-4 and IL-13 to HUVECs. Receptor binding analysis of radiolabeled IL-4 (*closed circles*) and IL-13 (*open circles*) was performed with resting HUVECs (*Resting*) or HUVECs stimulated with 1 ng/ml TNF-α for 24 h (*TNF-α*). Data were analyzed with the computerized weighted least-square curve fitting software described by Munson and Rodbard (26).

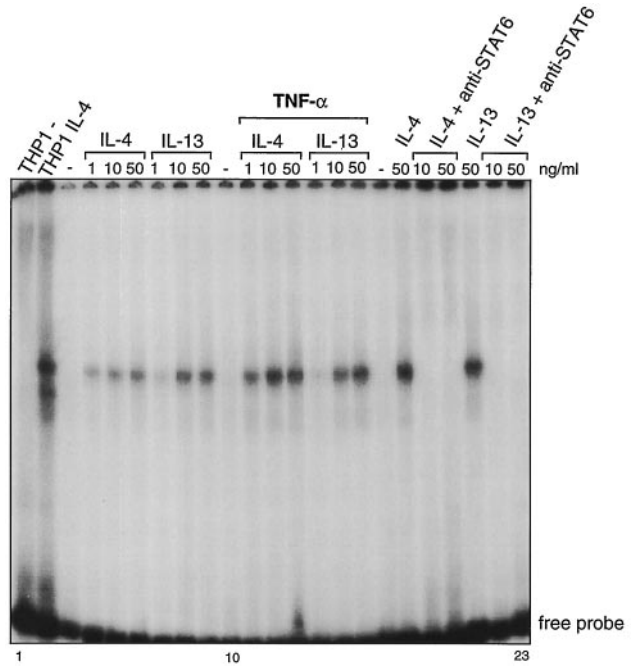

FIG. 6. **TNF treatment enhances the IL-4 and IL-13-induced IL-4-STAT (STAT-6) activation.** HUVECs were left untreated (*lanes 3–9*) or treated with 1 ng/ml TNFα for 18 h (*lanes 11–16*) before they were stimulated for 15 min with IL-4 or IL-13 at indicated concentrations. Both the IL-4- and the IL-13-induced shift complex can be supershifted with antisera against Stat6 (*lanes 19, 20, 22*, and *23*). Supershift controls in *lanes 18* and *21* are from TNF-activated HUVEC costimulated with IL-4 or IL-13, respectively. Resting and IL-4-activated THP-1 were used as a control (*lanes 1* and *2*).

IL-13 binding data derived from TNF-activated HUVECs only fitted the one-class binding site model with 856 ± 171 sites/cell and K_d value of 35 ± 7.7 pM. These data indicate that TNF-α significantly up-regulates the number of IL-4Rα but not that of the IL-13Rα subunits. The resulting corrected subunit stoichiometry and in turn increased heterodimerization of the receptor complex may explain the enhanced number of high affinity IL-13Rs.

TNF Treatment Enhances the IL-4- and IL-13-induced IL-4-STAT (STAT-6) Activation—Both IL-4 and IL-13 have been shown to signal through the activation of Stat6 (20, 23). We therefore tested for Stat6 activation by EMSA. In Fig. 6 both cytokines activate Stat6 to a similar extent. Pretreatment of HUVECs with TNF-α results in enhanced Stat6 activation. Supershifting experiments with antibodies against the various STATs identified the IL-4- and IL-13-induced shift as Stat6-specific shift.

Priming with TNF-α Increases IL-4-induced VCAM-1 Expression—In our previous work, IL-4 was shown to induce a variable but significant number of HUVECs to express VCAM-1 (8). In contrast TNF-α caused strong VCAM-1 expression in all HUVECs. Of interest to this study, VCAM-1 induction in response to TNF-α combined with IL-4 or IL-13 was more than additive (8). In Fig. 7, we primed HUVEC monolayers with TNF-α for 30 min before VCAM-1 expression was determined in the presence or absence of IL-4. Priming with TNF-α led to an enhanced IL4Rα expression (data not shown). After 16 h, non-primed HUVECs partially expressed VCAM-1 in response to IL-4, whereas TNF-α priming caused only marginal VCAM-1 induction. However, TNF-α priming in combination with IL-4 revealed an additive increase of the VCAM-1-expressing HUVEC population.

DISCUSSION

It has previously been shown that TNF-α has the ability to induce the expression of the IL-4 receptors on a mouse sarcoma cell line (43). In our study with HUVEC we demonstrate similar induction kinetics of the IL-4Rα, however at a much lower concentration of TNF-α. Furthermore, this observation could be extended to other proinflammatory stimuli such as lipopolysaccharide and IL-1. In stimulation experiments with TNF-α mutants selective for the 55- or 75-kDa TNF-α receptors, specific p55 TNFR-mediated effects were reported to cause endothelial activation (38) such as the induction of E-selectin, ICAM-1, and VCAM-1. Solely, the induction of α_2/β_1 integrin was shown to be mediated through both TNF receptors (37). In the present study, the up-regulation of the IL-4Rα was clearly under the control of the p55 TNFR, further confirming that the p55 TNFR is a major mediator of endothelial activation. Our data also support the TNF-α-dependent activation of a protein tyrosine kinase, as shown with the specific $pp60^{src}$-tyrosine kinase inhibitor herbimycin A (42).

IL-4 and IL-13 share a number of biologic activities on different leukocytic cells (4) and induce markedly similar expres-

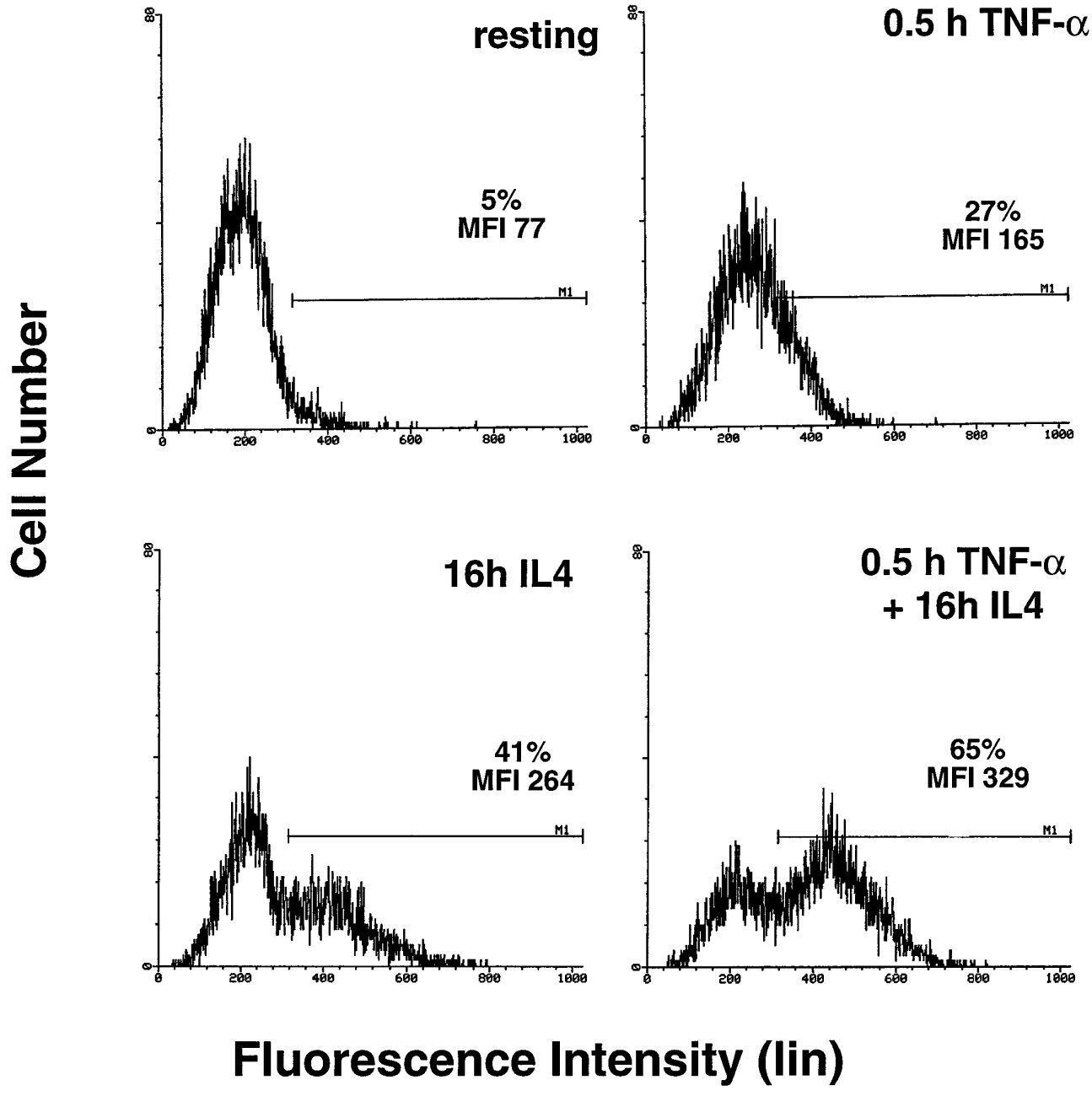

FIG. 7. **Induction of VCAM-1 expression on HUVECs.** HUVEC monolayers were either pulse-stimulated with TNF-α (1 ng/ml) for 30 min or incubated with IL-4 at 20 ng/ml for 16 h alone or in combination. Control monolayers were kept in medium alone (*resting*). Flow cytofluorometry for VCAM-1 expression was determined at the end of the 16-h incubation time. Corrected MFI and the immunopositive population (given in percent) within the margin (M1) are indicated from one representative experiment using the same batch of endothelial cells.

sion of VCAM-1 on HUVECs in culture (6). Recently, we have characterized on endothelial cells the receptor subunits for IL-4 and IL-13, which form a heterodimeric complex consisting of the IL-4Rα and a 75-kDa IL-13-binding protein and lack the expression of the γ_c (8). In that study, high concentrations of cold IL-4 displaced radiolabeled IL-13 from its binding to HUVECs and cold IL-13 competed for binding of radioactive IL-4. Such cross-competition suggests that IL-4 and IL-13 share the subunits of a heterodimeric receptor complex. The cross-linking experiments in this study show that TNF treatment did not modify the subunit composition of the receptor complex.

Of importance by flow cytofluorometry and RT-PCR analysis, TNF-α treatment did not reveal any induction of the γ_c. Nevertheless, binding of biotinylated IL-4 and flagged IL-13 to TNF-α-activated HUVECs was significantly enhanced. Therefore, receptor binding assays with radiolabeled IL-4 and IL-13 were performed to further analyze the qualitative changes in the IL-4·IL-13R complex. Previous receptor binding studies with resting endothelial cells, calculated according to a one-class binding site model, revealed a striking imbalance of the receptor subunits with a 2–3-fold excess of the IL-13R subunit (8). The use of a two-binding site model revealed for both cytokines ~200 high affinity receptors and a second receptor population for IL-13 with considerably lower affinity. TNF pretreatment significantly increased the number of IL-4 binding sites to about 600, whereas the K_d value did not change. Thus, TNF stimulation equilibrates the subunit ratio by solely up-regulating the number of IL-4Rα. The resulting 1:1 stoichiometry enhances receptor subunit dimerization causing increased numbers of high affinity IL-13Rs, as IL-13 binds now to

a single class of receptors with a similar high affinity as for IL-4. Recently, Leonard *et al.* (44) proposed a model for the IL-4R in which IL-4 may bind to IL-4Rα with low affinity before either the γ_c or the IL-13R are recruited. The resulting heterodimeric complex is proposed to form a high affinity receptor. Complex formation of a primary binding subunit, the α-chain, with a secondary affinity triggering subunit would explain the observed cross-competition of both cytokines. Recently, Hilton *et al.* (11) have cloned the mouse IL-13Rα. Its expression in various cells allowed the step by step validation of the above model. Here we first demonstrate the efficiency of the Leonard model on the basis of a regulatory mechanism in differentiated human cells.

Very recently Caput *et al.* (12) cloned and characterized a human IL-13-binding protein structurally related to the IL-5R α-chain. Single chains of the receptor, when transfected in COS-7 cells, are shown to bind IL-13 with high affinity. In cotransfection experiments with IL-4Rα heterodimerization and the related cross-competition were minimal. However, in HUVECs heterodimerization of IL-4R and IL-13R and ligand cross-competition were prominent, whereas single chain IL-13 receptors bound IL-13 with low affinity. These compelling differences suggest that a different kind of IL-13R is expressed in HUVECs. Indeed, by RT-PCR a transcript with close homology to the mouse IL-13R (11) could be amplified from HUVEC mRNA preparations. Sequence analysis of a related full-length cDNA clone revealed that the RT-PCR product represents a transcript with close homology to the above-mentioned mouse IL-13R.[2] The fact that the IL-13Rα message was not induced by TNF-α supports the concept of a regulatory induction of the IL-4Rα.

VCAM-1 expression on endothelial cells has been found to be the most significant function induced by IL-4 and IL-13 (5, 6). Unfortunately, TNF-α is a prominent inducer of VCAM-1 (45). However, VCAM-1 induction was more than additive when HUVECs were pretreated with a combination of TNF-α and IL-4 or IL-13 (8). Moreover, short time priming with TNF-α, which results in suboptimal VCAM-1 induction but fully enhanced IL-4Rα expression, increased in turn IL-4-mediated VCAM-1 expression (Fig. 7). Combined effects of TNF-α and IL-4 on VCAM-1 expression have been reported to be due to activation of VCAM-1 gene transcription by TNF-α and stabilization of the resultant VCAM-1 transcripts by IL-4 (46). IL-4 was found to exert its transcriptional effects on the VCAM-1 gene by an NF-κB-independent mechanism, which does not exclude the transcriptional contribution of other upstream *cis*-acting sequences nor the influence of posttranscriptional mechanisms (47). Many activities of IL-4 are shown to be mediated by the activation of STAT transcription factors (for review refer to Ref. 48). In hematopoietic cells, IL-4 specifically activates Stat6. Recently, Palmer-Crocker *et al.* (49) reported that in HUVECs IL-4 and IL-13 activate the JAK2 tyrosine kinase and Stat6. We studied Stat6 activation in order to discriminate between TNF-α and IL-4·IL-13-mediated effects. Our data support dose-dependent Stat6 activation in response to IL-4 and IL-13. Moreover, TNF-α treatment clearly enhanced the IL-4- and IL-13-mediated Stat6 activation. These data provide convincing evidence that the TNF-α-induced IL-4·IL-13R complex has an improved signaling capability. Since STATs are transcriptional activators, increased Stat6 activation may enhance transcription of different IL-4 and IL-13 target genes including VCAM-1. However, no data are yet available reporting a Stat6 consensus sequence in the VCAM-1 promoter.

Regarding the endothelial barrier it is tempting to speculate that the proinflammatory T_H1-type cytokines, which are characteristic for an acute inflammation with massive neutrophil infiltration, provide means for a switch to a chronic inflammatory state by increasing the expression of T_H2-type cytokine receptors.

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