Interferon α but Not Interleukin 12 Activates STAT4 Signaling in Human Vascular Endothelial Cells*

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STAT4 signaling, activated by either interleukin 12 (IL12) or interferon α (IFNα), promotes TH1 responses in CD4+ T cells. Vascular endothelial cells (EC) may also become polarized in response to various cytokines, favoring recruitment and activation of TH1 or TH2 effector cells. Here we have investigated the role of the STAT4 pathway in EC. Cultured human umbilical vein EC (HUVEC) express low levels of STAT4, which may be tyrosine-phosphorylated by treatment with IFNα but not IL12. This is because HUVEC lack both subunits of the IL12 receptor (IL12Rβ1 and IL12Rβ2), even following treatment with various cytokines. IL12 phosphorylation of STAT4 can be observed in HUVEC that have been transduced to express the IL12R. To identify STAT4-induced genes we pursued three approaches: analysis by DNA microarray and quantitative RT-PCR (Q-PCR) of the IL12 responses in IL12R-transduced EC; analysis by Q-PCR of IFNα responses in STAT4-overexpressing EC; and analysis of IFNα responses in U3A neuroblastoma cell lines that express either STAT1 or STAT4, but not both. In all three instances we observe STAT4-mediated induction of the chemokine monocyte chemoattractant protein 1 (MCP1) and suppressor of cytokine signaling 3 (SOCS3) mRNA, and we confirm the production of each protein in both IL12R-transduced EC and STAT4-transduced U3A cells. These observations reveal that there is a STAT4 response of EC, activated by IFNα but not IL12, and that it may modulate the pro-inflammatory behavior of EC.

Vascular endothelial cells (EC)1 are central to the development of inflammatory processes. In the resting state EC do not normally interact with circulating leukocytes. However, in response to inflammatory stimuli EC express cell surface adhesion molecules including E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) that allow for tethering and firm attachment of leukocytes, and ultimately their transmigration into the underlying tissues. The process of leukocyte recruitment by EC has been extensively reviewed (1–3). In general, the inflammatory response promoted by cytokines such as tumor necrosis factor (TNF), or bacterial products, such as lipopolysaccharide (LPS), predominantly involves recruitment of neutrophils. However, the pro-inflammatory functions of human EC can be modulated by cytokines such as IFNγ, a Th1 cytokine, and IL4 and IL13, both Th2 cytokines, to favor recruitment of other cell types. Treatment of cultured EC with IFNγ enhances expression of E-selectin and ICAM-1 (4), and the production of chemokines MIG, IP-10, and I-TAC (5, 6), all of which may favor the recruitment of macrophages and possibly Th1 CD4+ lymphocytes. In contrast, IL4 treatment of EC attenuates E-selectin (7) but promotes VCAM-1 expression (8) and leads to the production of chemokines such as eotaxin-3, which favors eosinophil recruitment and a Th2-like pattern of inflammation (9, 10). The effects of IFNγ are mediated by the transcription factor STAT1 (signal transducer and activator of transcription 1) (11), and those of IL4 (and the related cytokine IL13) by STAT6 (12). Another member of the STAT family, STAT4, which is activated by IL12 and IFNα, also contributes to Th1 responses (12).

STAT4 was first identified based on homology to other STAT proteins, and has been most extensively investigated as the mediator of IL12 responses in both human and murine T lymphocytes (12–14). IL12 binding to the IL12 receptor (IL12R) leads to dimerization of the two receptor subunits (IL12Rβ1 and IL12Rβ2) and activation of the receptor-associated tyrosine kinases Jak2 and Tyk2. These kinases then specifically phosphorylate tyrosine residues on the IL12R intracytoplasmic regions that allow for STAT4 binding and subsequent phosphorylation of STAT4 on tyrosine residue 693. Phosphorylated STAT4 dissociates from the receptor, dimerizes, translocates to the nucleus, and binds to STAT target sequences in IL12-responsive genes (15–18). STAT4 binding to 5′-upstream sequences of the murine IFNγ and human perforin genes has been demonstrated (19, 20), and DNA microarray studies in murine lymphocytes have identified more than 100 genes activated by IL12 whose expression is either directly or indirectly STAT4-dependent (21). STAT4 can also be activated by type 1 interferons. At least in humans, STAT4 is recruited to the IFNα receptor (IFNAR) indirectly via receptor-associated STAT2, leading to STAT4 tyrosine phosphorylation and ultimately the activation of STAT4-dependent genes as above (22, 23). The STAT4 signaling pathway plays a role in the polarization of CD4+ cell responses to an IFNγ-secreting Th1 phenotype, and STAT4-deficient mice have impaired Th1 responses (24–26).

In addition to T lymphocytes, STAT4 is also expressed by B
lymphocytes (27), NK cells (16), dendritic cells (DC), monocye,
cytophages, and macrophages (28, 29). IL12-induced STAT4 activ-
ation in both B and NK cells stimulates IFNγ secretion. Imma-
ture DC and resting monocytes or macrophages express little
STAT4. Upon DC maturation or monocyte/macrophage activa-
tion, for example by LPS, STAT4 expression is up-regulated
and mediates IL12- or IFNα-induced IFNγ secretion. Human
monocytes activate STAT4 in response only to IFNα, and not
IL12 (28). Two reports have suggested that STAT4 may be
expressed in non-lymphoid or non-myoeloid cells. Specifically,
STAT4 tyrosine phosphorylation was induced by cell surface
binding of sublytic terminal complement complex in human
aortic EC (30), and by treatment of human vascular smooth
muscle cells (VSMC) with urokinase (31). There are also some
reports that both EC and VSMC may respond to IL12. In one
study HUVEC were pretreated with IL12 prior to co-culture
with allogeneic CD4+ T lymphocytes, and in the presence of the
polyclonal mitogen phytohemagglutinin (PHA), IL12-treated
EC enhanced IFNγ production by the T cells, compared with
untreated EC (32). A second study demonstrated that primary
VSMC cultures treated with the combination of IL12 and IL18
could secrete IFNγ (33). However, it is not known whether
either IL12 or IFNα can activate a functional STAT4 signaling
pathway in either human EC or VSMC, or whether STAT4
activation leads to the induction of gene expression in either
cell type.

In this report we demonstrate that human EC do express
STAT4, but not the IL12 receptor. IFNα induces STAT4 tyro-
sine phosphorylation, as does IL12 in HUVEC transduced to
express both subunits of the IL12R. STAT4 signaling results in
sine phosphorylation, as does IL12 in HUVEC transduced to
produce PHA-activated EC in 1 µg/ml PHA for 3 days, supplemented with 10 units/ml IL2 (R&D systems) on
day 2.

The Phoenix-Ampho packaging cell line (a kind gift of Dr. G. Nolan,
Stanford University, Stanford, CA), and U3A cells (a kind gift of Dr. G.
Sulveci, Cleveland Clinic Foundation) were cultured in RPMI
modified Eagles medium (Invitrogen) containing 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM/L-t-glutamine. To
produce PHA-activated EC, 5 × 10^6 PHA were cultured in 1 µg/ml
PHA for 3 days, supplemented with 10 units/ml IL2 (R&D systems) on
day 2.

DNA Constructs and Retroviral Transduction—cDNAs encoding hu-
mans IL12Rβ1 and IL12Rβ2 subcloned into the vectors pBluescript II
SK+ and SK−, respectively, were purchased from ATCC (Manassas,
VA). The coding sequences flanked by HindIII (5') and NotI (3') sites
were amplified using the primers 5’-CCCCAGCTTGGTGAATAAGAAGAGCGCCATTGAGGCCGCCCCCTGCAGTTCAACTTCCATCTTGCCCCTGTC-3’ for IL12Rβ1 and 5’-CCCC
AACGTTGGATAAGAAGACCGGCATGCACATTTCTTATTGACC-3’ and 5’-ATAGTTTAACGCCGACATTCAATATGACCAGGAGTG-3’ for IL12Rβ2 (enzymes underlined, start and
stop codon in bold), and subcloned into the LZRspBMN-Z retroviral vector. Primes to amplify the coding region of STAT4 from a plasmid kindly provided by Dr. K. Mahboubi and Dr. A. Bothwell (Yale
University, New Haven, CT).

LZRspBMN-Z constructs were transfected into Phoenix-Ampho cells using Lipofectamine 2000 (Invitrogen) following the manufacturer’s
instructions. Purified recombinant cells were selected on condition either M199 (containing 10% FCS and 2 mM L-glutamine
but without ECGS, heparin or antibiotics) for HUVEC transduction
or Dulbecco’s modified Eagle’s medium containing 10% FCS and 2 mM L-glutamine for U3A cell transduction. Collection of virus-conditioned
medium and transduction of primary HUVEC cultures were performed
as previously described (36). For transduction of U3A cells, the cells
were grown in virus-conditioned medium containing 8 µg/ml polybrene (Sigma) for 24 h and then passaged once prior to use.

Immunoblotting, Immunoprecipitation, and ELISA—Lysis of confluent
U3A cells and HUVEC cultures grown in 6-well or 10-cm diameter
tissue culture dishes (Falcon, BD Biosciences), sample preparation, and
immunoblotting were performed exactly as described previously (36).
For immunoprecipitation, confluent cultures in 10-cm plates were lysed
in 1 ml of ice-cold radioimmune precipitation assay buffer (PBS con-
taining 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 µM
epbiole, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml benzamidine, 1
µm sodium orthovanadate, and 1 mM sodium fluoride) and clarified by
centrifugation at 10,000 × g for 10 min. Lysates were preclared by
incubation with 4 µg of goat IgG (R&D Systems) and 50 µl of washed
protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C. Following
centrifugation to remove the beads, the lysates were incubated with
4 µg of STAT4 antibody and 50 µl of washed protein G-Sepharose
at 4 °C overnight. The Sepharose beads were recovered by brief centrifuga-
tion and washed five times with PBS and resuspended in 50 µl of
SDS-PAGE sample buffer as previously described (36).

Antibodies were used for immunoblotting at the following concen-
trations: STAT4 0.2 µg/ml; phosphotyrosine STAT4 4 µg/ml; STAT1,
STAT3, phosphotyrosine STAT1, and phosphotyrosine STAT3 were
used at 1:1000 the concentration supplied by the manufacturer; β-actin
was used at 7 µg/ml; and SOCS3 at 1 µg/ml. STAT4 was pro-
duced intestinal heparin (Sigma). Confluent cultures were serially passaged
by trypsinization and used for experiments at the second or third
subculture. The culture medium was changed at least 24 h prior to any
cytokine treatment. Cytokines were added directly to the cultures with-
out further change of media.

Human dermal microvascular endothelial cells (HDMEC) were iso-
lated from discarded human skin, purified using anti-CD31 mini MACS
beads (Miltenyi Biotec, Auburn, CA) and cultured in EGM2-MV growth
medium (Clonetics, San Diego, CA) as previously described (35).
Human aortic and coronary artery VSMCs were isolated as previously
described (36), and cultured in M199 containing 20% FCS, 100 units/ml
penicillin, 100 µg/ml streptomycin, and 2 mM/L-t-glutamine. Per-
ipheral blood mononuclear cells (PBMCs) were obtained by leuka-
pheresis of adult volunteer donors, and further purified by centrifuga-
tion over lymphocyte separation medium (Organon Teknika, Durham,
NC) according to the manufacturer’s instructions. Isolated PBMC were
cultured in RPMI 1640 medium containing 10% FCS, 100 units/ml
penicillin, 100 µg/ml streptomycin, and 2 mM/L-t-glutamine. To
produce PHA-activated PBMC, 5 × 10^6 PBMC were cultured in 1 µg/ml
PHA for 3 days, supplemented with 10 units/ml IL2 (R&D systems) on
day 2.

Human IL12Rβ1 and IL12Rβ2 were isolated at the second or third
subculture. The culture medium was changed at least 24 h prior to any
cytokine treatment. Cytokines were added directly to the cultures with-
out further change of media.
facturer’s instructions. The primers used are shown in Table I. Quantitative real-time PCR (Q-PCR) for MCP1, TAP1, SOCS3, and GAPDH was performed exactly as described (36), using primers shown in Table I. All samples were analyzed in triplicate, and the results expressed as fold induction compared with baseline.

**DNA Microarray Analysis**—Fluorescently labeled probes were prepared from 50 μg of total RNA, isolated as above. cDNA was synthesized using SuperScript II RNase H−reverse transcriptase (Invitrogen) in a volume of 40 μl using the manufacturer’s buffer but containing 0.5 mM dATP, dGTP, and dCTP, 0.2 mM dTTP and 0.2 mM aminoallyl dUTP in PERM buffer, and flow cytometry were carried out as above.

**Q-PCR**—Total RNA was extracted from all cell types using guanidinium thiocyanate and chloroform as described (36) and treated with RNase-free DNase (Amersham Biosciences). Once the reaction was complete, the template RNA was hydrolyzed by incubation with 50 mM EDTA and 100 mM sodium hydroxide at 65 °C for 20 min. Following extraction with phenol/chloroform the cDNA was concentrated to a volume of 6 μl using a Micropartitioner (Amersham Biosciences), 4°C, washed a further two times and analyzed on a FACSort (Becton Dickinson). The probe mixture was heated to 94 °C for 2 min and then cooled to 55 °C. The probe was then applied to a DNA microarray comprising 16,000 70-mer oligonucleotide spots prepared by the Yale Keck Foundation Biopolymer Resource Laboratory. The probe was covered with a coverslip and incubated overnight at 55 °C. After removing the coverslip the slide was washed in 2× SSC, 0.2% SDS, and 0.2× SSC, each for 12 min at room temperature. The microarray was scanned by the Yale/Keck facility and analyzed using GenePix software (Axon Instruments, Union City, CA).

**Flow Cytometry**—For cell surface immunostaining, HUVEC, or HDMEC were washed twice with Hanks-buffered saline solution (HBSS) and incubated with trypsin/EDTA for 1 min. Detached cells were collected by centrifugation at 1000 × g for 5 min, washed twice with ice-cold PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS/BSA), and incubated with either IL12Rβ1 or IL12Rβ2 antibodies or isotype controls at 2 μg/ml in PBS/BSA for 2 h at 4 °C. After two further washes cells were incubated with 2 μg/ml PE- and fluorescein isothiocyanate-conjugated secondary antibodies in PBS/BSA for 1 h at 4 °C, washed a further two times and analyzed on a FACSort using CellQuest software (BD Biosciences). For intracellular immunostaining of STAT4, cells were detached as above and washed twice with ice-cold PBS before fixation with 2% paraformaldehyde for 15 min at room temperature. After a further two washes in PBS, cells were permeabilized for 15 min in PERM buffer (PBS containing 0.1% saponin, 1% fetal bovine serum and 0.1% sodium azide), washed once in PERM buffer and incubated with 1 μg/ml anti-STAT4 or goat IgG (R&D Systems). Further washes and incubation with secondary antibody, all in PERM buffer, and flow cytometry were carried out as above.

**RESULTS**

**IFNα, but Not IL12 or IFNγ, Induces STAT4 Tyrosine Phosphorylation in Cultured HUVEC**—IL12 or IFNα-mediated polarization of T cell immune responses to a Th1 phenotype depends on activation of the transcription factor STAT4. To determine whether this pathway is active in EC, we first investigated whether HUVEC express STAT4, and whether treatment with either IL12 or IFNα could induce STAT4 tyrosine phosphorylation. Because of its low abundance, we were unable to detect STAT4 by direct immunoblotting. To increase sensitivity we used immunoprecipitation with an affinity-purified polyclonal antibody, followed by immunoblotting, either with the STAT4 antibody or a phosphotyrosine-specific (Tyr693) STAT4 antibody. Fig. 1A shows that STAT4 could be immunoprecipitated from both HUVEC and PBMC. Treatment of both cell types with IFNα resulted in tyrosine phosphorylation of STAT4, which was transient in HUVEC when compared with PBMC. In contrast, IL12 induced STAT4 tyrosine-phosphorylation only in PBMC and not in HUVEC (Fig. 1A). Phosphorylation of STAT4 in HUVEC is specific to type 1 IFN, and not induced by treatment with IFNγ (Fig. 1B), although both interferon types induce STAT1 tyrosine phosphorylation (Fig. 1B). Parallel experiments using HDMEC and VSMC demonstrated STAT4 expression and tyrosine phosphorylation by IFNα in HDMEC but not in aortic or coronary artery VSMC (data not shown). These results demonstrate that cultured EC express STAT4, and that there appears to be a STAT4 signaling pathway in EC that may be activated by IFNα.

**FIG. 1. STAT4 expression and tyrosine phosphorylation in HUVEC.** A, HUVEC (grown to confluence in 10-cm tissue culture plates) or PHA-activated PBMC (5 × 105) were treated with either IFNα (5 ng/ml) or IL12 (10 ng/ml) for the times indicated. B, HUVEC were incubated with either IFNα (5 ng/ml) or IFNγ (40 ng/ml) for the times indicated. C, HUVEC were grown to confluence either with or without IFNγ (40 ng/ml) for 72 h and then stimulated with IFNα at the doses shown for 30 min. STAT4 and phospho-STAT4 were analyzed by immunoprecipitation of cell lysates with anti-STAT4 antibody followed by immunoblotting for either STAT4 or phospho-STAT4. STAT1, phospho-STAT1, and actin were analyzed by immunoblotting of whole cell lysates. Data are from one of at least three representative experiments.

| Table I | PCR primers used in this study |
|---------|--------------------------------|
| Target  | Primers (5′ → 3′)              |
| IL12Rβ1 | CCGAGAAACCCCTCCACG             |
|         | GCAGGGCAATTTGCCCCCA            |
| IL12Rβ2 | CTGAAACTGCTTGATCAA             |
|         | GTGCTCAATGATTACCTCC            |
| β-Actin | TCGATTCCTTCGAGCTCTC            |
| MCP1    | CAAAAGTGATCTTGCTGTATAT         |
| SOCS3   | GGGCACCCTCTTGACATC             |
| TAP1    | ATGCTACTGGTCGAGAATCT           |
| GAPDH   | GAAGGAGCAGCATTTGG              |
|         | ATGCGAGCAGCTGTTATTC            |

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Basal expression of STAT4 in both resting lymphocytes and monocytes is up-regulated upon activation of T cells, for example by PHA (16), and of monocytes by LPS or IFNγ (28). We next investigated whether STAT4 expression in HUVEC could be induced by similar treatments. Incubation of HUVEC with IFNα, LPS, IL6, oncostatin M, or VEGF for 24 or 72 h neither enhanced nor suppressed STAT4 expression (data not shown). However, 72 h treatment with IFNγ both reduced total STAT4 levels and attenuated STAT4 tyrosine phosphorylation in response to IFNα (Fig. 1C). IFNα-induced STAT1 phosphorylation was unaffected by IFNγ pretreatment.

**HUVEC Transduced to Express the IL12 Receptor Phosphorylate STAT4 and STAT3 in Response to IL12—HUVEC may be unable to respond to IL12 because they do not express one or both of the IL12R subunits, or because they lack downstream components of IL12R-mediated signaling. To investigate the first possibility, we performed RT-PCR on RNA extracted from HUVEC and PBMC. Both IL12Rβ1 and IL12Rβ2 mRNA could be detected in PBMC, but neither in HUVEC (Fig. 2). This analysis was repeated on 3 HUVEC cultures, each derived from 2 donors. In no case could either IL12R subunit be identified. Similarly, we could not identify mRNA for either IL12R subunit in isolates of cultured HDMEC (not shown). We also failed to find IL12R subunits by RT-PCR and flow cytometry on both HUVEC and HDMEC that were either untreated or treated with IFNγ, TNF, both in combination, LPS or IFNα (data not shown).

We next determined whether IL12 could induce STAT4 tyrosine phosphorylation in HUVEC transduced to express the IL12 receptor. Primary cultures of HUVEC were transduced to express both IL12Rβ1 and IL12Rβ2 or, as a control, EGFP. Cell surface expression of each subunit was analyzed by flow cytometry after 4 rounds of retroviral transduction. More than 95% of the HUVEC expressed either IL12R subunit, and 84% expressed both (Fig. 3A). Comparable frequencies of EGFP expression were observed in the control transduced cells (not shown). The response of these cells to IFNα and IL12 was analyzed by immunoprecipitation of cell lysates followed by immunoblotting for STAT4 and phospho-STAT4, or by immunoblotting of cell lysates for STAT1, STAT3, and phospho-STAT1 and 3. Control EGFP-EC responded to IFNα with tyrosine phosphorylation of STATs 1, 3, and 4, but did not respond at all to IL12 (Fig. 3B). In contrast, IL12 treatment of IL12R-EC induced tyrosine phosphorylation of both STAT4 and STAT3, but not STAT1 (Fig. 3B), consistent with the reported action of IL12 on CD4+ T cells (15, 17). IFNα treatment of IL12R-EC induced phosphorylation of STATs 1, 3, and 4, although the extent of STAT1, and particularly STAT3, phosphorylation was reduced in comparison to IFNα treatment of EGFP-EC (Fig. 3B). This attenuated IFNα response in IL12R-EC is likely because of competition for a limited supply of the receptor-associated tyrosine kinases Tyk2 and Jak2 between the endogenous IFNα receptor and the overexpressed IL12R. These results demonstrate that expression of both subunits of the IL12 receptor in EC is sufficient for IL12-mediated STAT4 tyrosine phosphorylation.

**Identification of SOCS3 and MCP1 as Potential STAT4-dependent Genes in HUVEC—Cellular responses to IFNα are characterized by the expression of many genes activated through the STAT1/STAT2/IRF9 pathway. For example, microarray analysis of mRNA expressed in EC has identified more than 200 genes induced at least 2-fold by IFNα (37). Our results suggest that some of these genes could be in part STAT4-dependent. To identify candidate STAT4-dependent genes in EC we made use of the IL12R-EC. Using DNA microarray techniques we compared the expression of genes in EGFP-EC with those in IL12R-EC following treatment of both cell lines with IL12 for 3 h. Only the genes encoding MCP1 and SOCS3 were consistently up-regulated, and none down-regulated, in 3 IL12R-EC lines, each derived from three different pooled donors. Thus the number of genes in EC regulated by STAT4 is remarkably limited. Up-regulation of these genes in IL12R-EC in response to IL12 was confirmed by Q-PCR (Fig. 4A). In three
experiments the median fold induction of MCP1 mRNA in IL12R-EC compared with EGFP-EC was 10-fold (range 6.8–15.6) and that of SOCS3 mRNA 4.8-fold (range 2.9–5.4). Both of these genes are also activated by IFNα/H9251 treatment of EC (Fig. 4B). In comparison to IL12 treatment of IL12R-EC, IFNα/H9251 treatment of EGFP-EC is a more potent inducer of both MCP1 and SOCS3, with peak mRNA induction at 1 h as opposed to 3 h (Fig. 4B). We also performed microarray studies on the STAT1-deficient U3A cell line transduced to express STAT4. These cells phosphorylate STAT4 in response to IFNα (shown in Fig. 6A). Although the STAT4 response of these cells is also limited, it appears greater than that observed in EC. In addition to MCP1 and SOCS3, the mRNA for SOCS1, cytokine-inducible SH2 domain-containing protein (CIS), CIS2, IL6, and the IL1 receptor (IL1R) were induced by IFNα in a STAT4-dependent manner (data not shown). However, we were unable to demonstrate IL12 induction of SOCS1, CIS, CIS2, IL6, or IL1R in IL12R-EC.

To be certain that the up-regulation of MCP1 and SOCS3 by IL12 in IL12R-EC is not caused by low level STAT1 or STAT1/2 activation undetectable by immunoblotting, we analyzed the expression of a marker STAT1- and STAT1/2-dependent gene, TAP1, in both EGFP-EC and IL12R-EC in response to IL12 and IFNα (Fig. 4C). IL12 was unable to induce TAP1 mRNA expression in either cell type, whereas IFNα up-regulated TAP1 expression in both. These results suggest that at least two genes, MCP1 and SOCS3, may be regulated in part by STAT4 (or STAT3) in EC exposed to IFNα.

The induction of MCP1 and SOCS3 mRNA by IL12 in IL12R EC is accompanied by increased synthesis of the corresponding proteins. Treatment of IL12R EC with IL12 leads to MCP1 secretion into the culture medium (Fig. 4D). There is no increase in MCP1 production above baseline in EGFP-EC treated with IL12, whereas IFNα-induced MCP1 secretion in EGFP-EC exceeds that induced by IL12 in IL12R-EC, consistent with the levels of MCP1 mRNA induction in each cell type. Similarly, IL12 treatment of IL12R-EC induces SOCS3 protein synthesis, but to a lesser extent than IFNα treatment of EGFP-EC (Fig. 4E). The response of IL12R-EC to IFNα is diminished, and we have speculated that this is because of competition for Jak kinases.

**Overexpression of STAT4 Enhances MCP1 and SOCS3 Expression in HUVEC**—IFNα treatment of EC activates STATs 1/2, 3, and 4. Consequently it is not possible to be certain that either MCP1 or SOCS3 are regulated by STAT4, and if so to what extent. We reasoned that if MCP1 and SOCS3 are at least in part STAT4-dependent then overexpression of wild-type STAT4 in EC should enhance expression of MCP1 and SOCS3 in response to IFNα. Primary cultures of HUVEC were transduced to express STAT4. After 4 rounds of retroviral transduction more than 90% of the EC overexpressed STAT4 measured by intracellular flow cytometry (Fig. 5A). Expression of STAT4
could now be readily detected by immunoblotting of cell lysates of STAT4-EC, but not of EGFP-EC (Fig. 5B), in which STAT4 could only be detected by immunoprecipitation (not shown). Upon treatment with IFNα there is tyrosine phosphorylation of STAT4 that, in contrast to wild-type EC, persists for more than 2 h following IFNα administration (Fig. 5B, compare with Fig. 1, A and B). Phosphorylation of both STAT1 and STAT3 in response to IFNα did not differ between EGFP-EC and STAT4-EC (Fig. 5B). Expression of TAP1, SOCS3, and MCP1 mRNA was analyzed by Q-PCR (Fig. 5C). IFNα induced comparable up-regulation of TAP1 mRNA in EGFP-EC and STAT4-EC. In contrast the expression of both SOCS3 and MCP1 was both enhanced and prolonged in STAT4-EC. These results confirm that MCP1 and SOCS3 can be regulated by STAT4 in EC.

**STAT4 Is Sufficient for IFNα-induced MCP1 and SOCS3 Expression in U3A Cells**—To provide further evidence that regulation of MCP1 and SOCS3 expression by IFNα can be STAT4-mediated, we made use of the U3A neuroblastoma cell line. These cells do not respond to IFNα because they do not express STAT1 (or STAT4) (22, 38), although they do express both STAT2 and STAT3. We generated retroviral transductants of U3A cells expressing either STAT1 (U3A-s1) or STAT4 (U3A-s4), both of which were expressed in more than 90% of the cells (not shown). Treatment with IFNα for 20 min induced comparable STAT3 tyrosine phosphorylation in U3A, U3A-s1, and U3A-s4 cells, and of STAT1 and STAT4 in U3A-s1 and U3A-s4 cells, respectively (Fig. 6A). IFNα-induced expression of TAP1, SOCS3, and MCP1 mRNA in each cell type is shown in Fig. 6, B–D. None of these genes was up-regulated in U3A cells, suggesting that, at least in this cell type, STAT3 activation alone is insufficient to induce either MCP1 or SOCS3 mRNA transcription. However, IFNα treatment of U3A-s4 cells potently induced SOCS3 (Fig. 6C) and MCP1 mRNA expression (Fig. 6D) and protein synthesis (Fig. 6, E and F). In contrast, IFNα treatment of U3A-s1 cells resulted in modest up-regulation of SOCS3 and negligible induction of MCP1, but substantial TAP1 expression. These results suggest that both SOCS3 and MCP1 mRNA can be induced by IFNα through a STAT4-dependent pathway in cell types other than HUVEC.

**DISCUSSION**

STAT4 signaling, activated by IL12 or IFNα, is central to the development of Th1-type cell-mediated immune responses (12). STAT4 activation in CD4+ T lymphocytes leads to the differentiation of IFNγ-secreting Th1 cells while suppressing the development of IL4-secreting Th2 cells (24). Similarly, STAT4 activation in both NK cells and monocytes leads to IFNγ secretion (29), and STAT4 is highly expressed in macrophages at sites of Th1 inflammation (28). The transcription factor STAT6 plays an analogous role in the development of IL4 and IL13-mediated Th2 responses. STAT6 is widely expressed (39–41), and activation of STAT6-dependent pathways in EC (10) and in epithelial cells of the respiratory tract (42) promotes Th2-like inflammation. In contrast, STAT4 signaling has been identified only in lymphoid and myeloid cells. We show here that STAT4 is also expressed in human EC, specifically both cultured HUVEC and HDMEC. In EC IFNα, but not IL12, induces STAT4 tyrosine phosphorylation, which may promote expression of both MCP1 and SOCS3.

Two previous reports have suggested that STAT4 may be expressed in non-immune cells. In one, STAT4 phosphorylation was induced by treatment of aortic EC with sublytic C5b-9 complement complex (30). In contrast to our own observations, phosphotyrosine STAT4 was detectable in unstimulated cells. Moreover, we have been unable to demonstrate STAT4 activation by heterologous rabbit complement in HUVEC. In a second report, treatment of VSMC with urokinase apparently

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*Fig. 5. Overexpression of STAT4 enhances SOCS3 and MCP1, but not TAP1, expression in HUVEC. A, HUVEC were retrovirally transduced to overexpress STAT4. After four rounds of transduction STAT4 expression was analyzed by intracellular staining and flow cytometry and compared with HUVEC transduced with empty vector (Control EC). IgG, polyclonal goat IgG control. HUVEC transduced with either EGFP or STAT4 were incubated with IFNα/H9251 U3A-s4 cells, respectively (Fig. 6A and B). Cell lysates were analyzed for STAT4, phospho-STAT4, phospho-STAT1, phospho-STAT3, and STAT3 by immunoblotting. C, TAP1, SOCS3, and MCP1 mRNA expression was analyzed using Q-PCR. Results are expressed as fold induction compared with cells untreated with cytokine in each group, and normalized to GAPDH as described under "Experimental Procedures." Similar results were obtained from two independently derived STAT4-transduced HUVEC lines.*

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*N. Torpey and J. S. Pober, unpublished observations.
induced both tyrosine phosphorylation and nuclear translocation of STAT4 (31). Again, phosphotyrosine-STAT4 was detectable in unstimulated cells. We have been unable to detect STAT4 in either aortic or coronary artery VSMC. One possible explanation for these discordant results concerns the antibodies used in these reports. In cell types with low levels or absent STAT4 expression, for example EC and VSMC, we found that immunoprecipitation with the STAT4 antibodies used in these studies, followed by immunoblotting for phosphotyrosine, yielded many nonspecific bands. Only by using a specific phosphotyrosine-STAT4 antibody were we able to obtain clean and reproducible results.

STAT4 is induced upon activation of resting T cells and maturation of monocytes and dendritic cells. This is not the case in EC, in which pro-inflammatory cytokines either had no effect on STAT4 or, in the case of IFNγ, actually suppressed both STAT4 expression and signaling. Furthermore, STAT4 activation in EC did not lead to the induction of genes associated with Th1-like responses, but instead to the expression of MCP1 and SOCS3. Thus STAT4 responses in EC are both quantitatively and qualitatively distinct to those described in other cell types. One caveat is that we were unable to isolate STAT4 signaling in IL12R-transduced EC, because STAT3 was also activated. SOCS3 is clearly STAT3-dependent in several cell types (36, 43), and may be so in EC. Strategies to knock down STAT4 expression in EC with siRNA have, to date, been unsuccessful in our hands. Nevertheless, enhanced expression of SOCS3 in both EC and U3A cells overexpressing STAT4 indicates that STAT4 can also regulate SOCS3 expression. A second caveat is that we have been unable to identify conditions under which EC express the IL12R. It is possible that such conditions exist in vivo. This is important because the STAT4 response of EC to IFNα is likely overwhelmed by the STAT1/2 response. In contrast, specific induction of SOCS3 and MCP1 by IL12 may have significant effects on EC pro-inflammatory functions.

SOCS3 is one of eight members of the suppressor of cytokine signaling family (44). SOCS proteins are rapidly induced by cytokines, and act as negative regulators of signaling by binding to either the receptors or receptor-associated JAK kinases.
Recently, SOCS3 has been shown to inhibit IL12-induced STAT4 activation by competing with STAT4 for binding sites on IL12Rβ2 (45). Thus it is not surprising that STAT4 signaling in EC should lead to SOCS3 expression as a classical negative regulatory feedback response. STAT4-mediated IL12 signaling is also inhibited by SOCS1 (46). IL12 treatment of IL12R EC did not lead to SOCS1 expression, although IFNα was able to induce SOCS1 in STAT4 transduced U937 cells. SOCS1 is potently induced by IFNγ (44), and it is likely that the impaired STAT4 phosphorylation we observed in IFNγ pre-treated EC is caused by SOCS1 induction. SOCS proteins may also inhibit responses to cytokines distinct from those that induced their synthesis. SOCS3 is particularly associated with inhibiting signaling by IL6 and related cytokines, all of which share gp130 as a common receptor subunit (44). In EC, treatment with both IL6 and IL11 appears to exert a cytoprotective effect, for example attenuating injury induced by antibody (47) or hydrogen peroxide (48). In these circumstances STAT4-induced SOCS3 may promote inflammation by inhibiting cytoprotective responses.

The chemokine MCP1 also contributes to pro-inflammatory functions of EC. MCP1 promotes firm adhesion of monocytes to EC monolayers under flow conditions (49), and in two models of atherosclerosis MCP1-deficient mice demonstrate reduced monocyte and macrophage recruitment in the vessel wall compared with controls (50, 51). MCP1 may also promote atherogenesis by stimulating proliferation and migration of VSMC (52). That there is a Tγ1-like STAT4-dependent pathway of MCP1 production in EC is consistent with the observation that in some circumstances, for example transplant arteriosclerosis, the development of vascular disease is dependent upon Tγ1 responses and IFNγ secretion (53). In some instances MCP1 has been associated with Tγ2- rather than Tγ1-like inflammatory responses. Mice deficient in MCP1 display impaired secretion of the Tγ2 cytokines IL4 and IL5, but normal IFNγ secretion, and have attenuated responses to pathogens that normally induce a Tγ2 response (54, 55). Nevertheless, MCP1 deficiency also abrogated inflammatory responses in murine experimental autoimmune encephalomyelitis (EAE), a Tγ1-mediated disease (56). Thus, although STAT4 activation in EC does not lead to the induction of classical Tγ1-type genes, the expression of MCP1 is consistent with a role in promoting Tγ1-like responses under appropriate conditions.

In this report we have shown that human EC express STAT4, and identified a STAT4-dependent signaling pathway activated by IFNγs and potentially IL12 that leads to the induction of SOCS3 and MCP1 mRNA and protein. We speculate that activation of STAT4 in EC contributes to inflammatory responses that may play a role in the development of vascular disease.

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REFERENCES

1. Springer, T. A. (1994) Cell 76, 301–314
2. Lucisancas, F. W., Ma, S., Nusrat, A., Parkos, C. A., and Shaw, S. K. (2002) Semin. Immunol. 14, 105–113
3. Pober, J. S. (1999) Immunol. Rev. 189, 225–232
4. Doukas, J., and Pober, J. S. (1990) J. Immunol. 145, 1727–1733
5. Rau, J., Malloy, A., Shah, T., Smith, B., Oaks, M., and Hosenpud, J. D. (2003) J. Immunol. 170, 1027–1074
6. Monaco, C., Andreaskos, E., Young, S., Feldmann, M., and Paleolog, E. (2002) Eur. J. Immunol. 32, 659–665
7. Bennett, B. L., Cruz, R., Lacoan, R. G., and Manning, A. M. (1997) J. Biol. Chem. 272, 10212–10219
8. Thernhill, M. H., Wollcrom, S. M., Mahouz, D. L., Laneheber, J. S., Kyan-Aung, U., and Haskard, D. O. (1991) J. Immunol. 146, 592–598
9. Shinkai, A., Yushise, H., Koike, M., Shoji, N., Nakagawa, S., Saito, A., Takeda, T., Imahepp, S., Kato, Y., Hanai, N., Anazawa, H., Kuga, T., and Nishi, T. (1999) J. Immunol. 163, 1602–1610
10. Cuvelier, S. L., and Patel, K. D. (2001) J. Exp. Med. 194, 1699–1709
11. Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002) Trends Immunol. 23, 96–101