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IFN-γ Inhibits Activation-Induced Expression of E- and P-Selectin on Endothelial Cells

Jennifer Melrose, Naoya Tsurushita, Gao Liu, and Ellen L. Berg

E- and P-selectin are cell surface lectins that mediate leukocyte-endothelial cell adhesion and thereby participate in neutrophil recruitment into inflammatory sites. E-selectin can be induced on endothelial cells by various activators, including TNF-α, IL-1β, and PMA. Induction of E-selectin is blocked by pretreatment of endothelial cells with IL-4 or TGF-β, both of which have antiinflammatory properties in vivo. In addition to its well-known proinflammatory activities, IFN-γ also has antiinflammatory effects in vivo, one of which is inhibition of neutrophil recruitment. To determine whether IFN-γ inhibits neutrophil recruitment by inhibiting adhesion molecule expression, the effect of IFN-γ on activation-induced cell adhesion molecule expression by cultured HUVEC was evaluated. Pretreatment of endothelial cells with IFN-γ for 24 to 72 h before 6- to 24-h activation with IL-1β, TNF-α, or PMA resulted in significantly reduced levels of cell surface E-selectin, although levels of ICAM-1 and VCAM-1 were the same or increased. The reduction of cell surface E-selectin levels under these conditions was reflected in reduced levels of E-selectin mRNA, indicating an effect at the transcription level or RNA stability. Interestingly, the increase of cell surface P-selectin expression due to IL-4 treatment of HUVEC was also inhibited by IFN-γ, while constitutive levels of P-selectin were not. These results suggest that the inhibition of neutrophil recruitment by IFN-γ in vivo may be due, in part, to the ability of IFN-γ to inhibit E- and P-selectin up-regulation. Furthermore, these findings emphasize the process of leukocyte recruitment as an important step through which IFN-γ can direct the character of inflammatory reactions. The Journal of Immunology, 1998, 161: 2457–2464.

The proinflammatory activities of IFN-γ are well known and include a central role in macrophage activation, induction, and enhancement of MHC class I and II Ags on a wide variety of cell types, differentiation of B cells, and regulation of the proliferation and function of activated T cells (reviewed in Ref. 1). IFN-γ production is a characteristic of Th1 cells, cells that drive delayed-type hypersensitivity reactions. Th1 responses are required for protection from intracellular pathogens and are most likely important for the development of certain autoimmune syndromes (2).

Despite understanding many of the proinflammatory activities of IFN-γ, the role of IFN-γ in autoimmune and inflammatory diseases has been difficult to elucidate. Conflicting data have been observed in many in vivo models. For example, IFN-γ treatment protects C57BL/6J mice from experimental allergic encephalomyelitis, which some describe as a model of multiple sclerosis; however, treatment of patients with IFN-γ has led to an exacerbation of the disease (3, 4). While Ab to IFN-γ delays the lupus-like nephritis in (NZB × NZW)F1 mice, it has no effect on the nephritis in the MRL/lpr-lpr mouse model of lupus (5, 6). Abs to IFN-γ also have disparate effects in the adjuvant arthritis model in rats, depending on when therapy is given (7, 8). Together these data have suggested that IFN-γ may have both anti- and proinflammatory activities, the interactions of which are not well understood (1, 4).

The ability of IFN-γ to modulate leukocyte recruitment is one function for which both anti- and proinflammatory activities have been ascribed. Although IFN-γ is a potent inducer of lymphocyte and monocyte recruitment (9, 10), several reports have suggested that IFN-γ may also block neutrophil recruitment (11–13). Several years ago, Hallmann, in collaboration with Jutila, Amento, and Butcher, observed that treatment of cultured endothelial cells with IFN-γ resulted in reduced neutrophil adherence to IL-1β-activated endothelial cells (14). To determine the mechanism by which IFN-γ inhibits neutrophil-endothelial cell adhesion, we tested the ability of IFN-γ to modulate the expression of endothelial cell adhesion molecules for neutrophils.

Materials and Methods

Cytokines and Abs

Human recombinant IFN-γ, TNF-α, IL-1β, and IL-4 were obtained from R&D Systems (Minneapolis, MN) and reconstituted, as suggested by the manufacturer, except for IFN-γ, which was reconstituted in 0.1% BSA in PBS. PMA was obtained from Clonetics (San Diego, CA). Human thrombin and mouse IgG1 and IgG2b myeloma proteins MOPC 21 and MOPC 141 were obtained from Sigma (St. Louis, MO). mAb E-1E4, anti-human E-selectin (murine IgG1), has been previously described (15). E-1E4 was biotinylated by labeling with long chain biotin, according to the method described by Pierce (Rockford, IL). AF-2 (murine IgG2b), a neutralizing mAb to human IFN-γ, was kindly provided by Nick Landolfi (Protein Design Labs, Mountain View, CA). Anti-human VCAM-1 from Coulter (Miami, FL); and human P-selectin, WAPS 12.2, from Endogen (Woburn, MA). J13C2.4 is a mouse IgG1 anti-HLA-DR Ab, generated in our laboratory, and purified from serum-free supernatants, as described (15).

Endothelial cell cultures

HUVEC were obtained from Clonetics. These cells were cultured in endothelial cell growth media (EGM; 2 Clonetics), containing bovine brain extract (12 μg/ml), human epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and 2% FBS in MCD1-131 medium, for one to eight passages, and subcultured with trypsin-EDTA, as described by the manufacturer. Experiments were

2 Abbreviations used in this paper: EGM, endothelial cell growth medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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performed by culturing HUVEC in 96-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ), in the presence of various cytokines or activators for the indicated times.

**Adhesion assays**

HL-60 cell binding to HUVEC was performed essentially as described (15, 16). Briefly, HUVEC were cultured in 96-well plates in EGM with or without IFN-γ for 24 h, then with or without PMA for an additional 4 h, washed, and resuspended in 50 µl/well assay buffer (10% adult bovine serum/10% normal rabbit serum/10 mM HEPES, pH 7.2/RPMI). Fluorescently labeled HL-60 cells were prepared as previously described (15) and resuspended in assay buffer containing 0.25 µg/ml anti-CD18 Ab, N9A, at 2 × 10^6 cells/ml. Assays were initiated by the addition of 50 µl HL-60 cells to HUVEC for a final volume of 0.1 ml, while plates were rotated at 40 rpm (Innova 200; New Brunswick, Edison, NJ). After 15 min at room temperature, unbound HL-60 cells were removed by washing four times with 0.2 ml RPMI/well. Bound cells were fixed by the addition of 0.1 ml 1% paraformaldehyde (Sigma) in PBS. Plates were analyzed with a Microplate Fluorometer (model 7620; Cambridge Technology, Watertown, MA), and the relative binding of HL-60 cells was determined by measuring the amount of fluorescence at 530 nm, using an excitation of 485 nm.

**Cell-based ELISAs**

Microtiter plates containing treated HUVEC were washed with 200 µl/well PBS and inverted until plates were dry. Wells were blocked by incubating with 200 µl 1% Blotto (Pierce) in PBS for 30 min. Primary Abs, including anti-E-selectin, anti-VCAM-1, anti-ICAM-1, anti-HLA-DR, anti-P-selectin, isotype control Abs, or biotinylated anti-E-selectin, were then added to washed plates at 0.5 to 2 µg/ml in 0.05% Blotto/PBS for 2 h. Plates were again washed in 0.05% Blotto/PBS, and 50 µl peroxidase-conjugated anti-mouse IgG (Promega, Madison, WI), diluted 1/3000, or peroxidase-conjugated streptavidin (Pierce), diluted 1/1000, in 0.05% Blotto/PBS, was applied for 1 h. After washing, 100 µl TMB substrate (Kirkgaard and Perry, Gaithersburg, MD) was added and color was developed for 5 to 10 min. A total of 100 µl 1 M H₂SO₄ was then added and absorbance was read at 450 nm (subtracting the background absorbance at 600 nm) with a Dynatech (Chantilly, VA) plate reader.

**Flow cytometry**

HUVEC were released from tissue culture plates by brief incubation in trypsin-EDTA (Clonetics). After washing cells in EGM, flow-cytometry experiments were performed by incubation of 5 × 10^5 cells in 0.1 ml FACS buffer (0.1% BSA, 10 mM NaCl in PBS) with 10 to 100 ng primary Ab for 1 h at 4°C. After washing, 50 µl of goat F(ab)₂ anti-mouse IgG-phycocerythrin conjugated (Biosource, Camarillo, CA), diluted 1/500 in FACS buffer, was added and incubated for 30 min before washing and fixation in 1% paraformaldehyde. Cells were analyzed with a FACSScan (Becton Dickinson, San Jose, CA), according to standard procedures.

**Quantitative RT-PCR**

The procedure for utilizing known concentrations of competitive DNA fragments in PCR reactions for precisely measuring mRNA levels (as described in Refs. 17–19) was obtained from Clonetch Laboratories (Palo Alto, CA). HUVEC were cultured in 24-well plates, as described above, in the presence of IFN-γ (0 or 250 ng/ml), for 48 h, and PMA (100 ng/ml) for an additional 4 h. Cells were solubilized by direct treatment with TRIzol reagent (Life Technologies, Gaithersburg, MD), and total RNA was prepared according to the manufacturer’s instructions. The first-strand cDNA synthesis was performed with SuperScript II RNase H− reverse transcriptase (Life Technologies) using random hexadeoxynucleotides (Pharmacia, Piscataway, NJ) as primer. PCR reactions contained a fixed amount of cDNA, various known concentrations of E-selectin, ICAM-1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) competitor DNAs, and appropriate primers. The reaction was performed with TaqPlus polymerase (Stratagene, La Jolla, CA) at 94°C for 5 s, 57°C for 5 s, and 72°C for 1 min with 30 cycles. Competitor DNA and PCR primers for GAPDH were obtained from Clontech, and result in native and mimic PCR products of 983 and 691 bp, respectively. The PCR primers for E-selectin were 5′-AC CTCACCAGGAAGCTATGAC-3′ and 5′-TCCCAAGATGAGGTA CACTGA-3′, which produce a 796-bp native PCR product; and those for ICAM-1 were 5′-ACATGCGACCCATG-3′ and 5'-AGAAG GAGCTGTTGCCATAG-3′, which produce a 673-bp native PCR product. Competitor DNAs for E-selectin and ICAM-1 were constructed by deleting 240- and 213-bp fragments, respectively, within the regions amplified by the above PCR primers from the corresponding cDNAs to yield mimic PCR fragments of 556 and 460 bp, respectively. The PCR products were separated on a 3% agarose gel (Life Technologies), and the intensities of native and mimic PCR products for each reaction (each containing a different amount of mimic DNA) was determined from an ethidium bromide-stained gel photograph, by densitometry using the computer program National Institutes of Health Image, version 1.51 (an updated version of which can be obtained through the Internet at: http://rsb.info.nih.gov/nih-image).

**Results**

**Effect of IFN-γ on HL-60 cell adhesion to HUVEC**

Neutrophils and HL-60 cells bind avidly to HUVEC that have been pretreated with one of a variety of activators, including TNF-α, IL-1β, but not IFN-γ (20). As first observed by Hallmann for neutrophil binding to IL-1β-activated HUVEC (14), Figure 1 shows that 24-h pretreatment of HUVEC with IFN-γ results in significantly reduced HL-60 cell binding to PMA-activated HUVEC.

**Modulation of endothelial cell adhesion molecule expression by IFN-γ**

To test whether activation-induced expression of endothelial cell adhesion molecules was affected by IFN-γ pretreatment, HUVEC were pretreated for 24 h with IFN-γ, then evaluated for the expression of E-selectin, ICAM-1, VCAM-1, or HLA-DR, or IL-1β, or IL-1α, or IL-1α activation, IFN-γ pretreatment further increased the levels of VCAM-1 or ICAM-1. In contrast, E-selectin expression due to PMA, IL-1α, or TNF-α activation was reduced significantly if HUVEC were first pretreated with IFN-γ (Fig. 2). This effect was observed both at 6 and 24 h following addition of activators. The inhibitory effect of IFN-γ on activation-dependent E-selectin expression was tested over a wide range of activator concentrations (Fig. 3). High concentrations of activators, up to 100 ng/ml PMA, or 25 ng/ml TNF-α or IL-1α did not overcome the inhibitory effect of IFN-γ pretreatment on E-selectin induction. The concentrations

**FIGURE 1.** Effect of IFN-γ pretreatment of HUVEC on activation-dependent HL-60 cell adhesion. Confluent HUVEC were treated with 250 ng/ml IFN-γ ■ or medium alone □ for 24 h, and then activated with PMA (100 ng/ml) or media (None) for an additional 4 h. Adhesion of fluorescently labeled HL-60 cells to treated HUVEC grown in 96-well plates was evaluated, as described in Materials and Methods. The relative number of HL-60 cells binding to HUVEC under the various conditions was determined by measuring the mean fluorescence intensity (MFI) in each well. The mean ± SD, n = 4, from a single representative experiment is shown. *Indicates p < 0.005; NS indicates not significant, comparing with and without IFN-γ.
of IFN-γ required to inhibit activation-dependent E-selectin expression were then compared with the concentrations of IFN-γ required to induce ICAM-1 or HLA-DR expression on HUVEC (Fig. 4). Similar concentrations of IFN-γ were found to be required to inhibit activation-induced E-selectin expression and to induce ICAM-1 and HLA-DR expression.

Neutralization of IFN-γ by anti-IFN-γ Ab, AF-2

The ability of IFN-γ pretreatment to inhibit activation-dependent E-selectin expression by HUVEC was evaluated in the presence or absence of a neutralizing mAb to IFN-γ, AF-2 (Fig. 5). In the presence of AF-2, but not an isotype control mAb, the inhibitory effect of IFN-γ on PMA-induced E-selectin expression was reversed, indicating that the effects observed with IFN-γ were not due to a nonspecific inhibitor present in the preparation of IFN-γ.

Time course of IFN-γ pretreatment effects

To determine the exposure time required for IFN-γ to inhibit activation-induced E-selectin expression, HUVEC were treated at the same time or 1, 2, or 3 days before activation with TNF-α, PMA, or IL-1β.

FIGURE 2. Effect of IFN-γ pretreatment on the expression of HUVEC endothelial cell adhesion molecules following 6- or 24-h activation by PMA, TNF-α, or IL-1β. HUVEC cells were cultured with 200 ng/ml IFN-γ or medium alone for 24 h, and then activated with PMA (1 ng/ml), TNF-α (0.5 ng/ml), IL-1β (1 ng/ml), or media alone (None) for an additional 6 (A) or 24 h (B). The levels of E-selectin, VCAM-1, ICAM-1, or HLA-DR were determined by a cell-based ELISA, as described in Materials and Methods. The relative expression levels of each adhesion molecule are indicated by the OD at 450 nm. The mean ± SD of triplicate samples in a single representative experiment is shown. **Indicates p < 0.005; *indicates p < 0.05, comparing with and without IFN-γ.

FIGURE 3. Effect of activator concentration on E-selectin expression on HUVEC pretreated with or without IFN-γ. HUVEC were cultured with 250 ng/ml IFN-γ or medium alone for 48 h, and then the indicated concentrations of PMA, TNF-α, or IL-1β were added for an additional 6 h. The level of E-selectin expression was determined by a cell-based ELISA, as described in Materials and Methods. The relative expression level of E-selectin is indicated by the OD at 450 nm. The mean ± SD of triplicate samples in a single representative experiment is shown. ** Indicates p < 0.005; * indicates p < 0.05, comparing with and without IFN-γ.
or IL-1α for 6 h, then evaluated for E-selectin (Fig. 6A) or ICAM-1 (Fig. 6B) expression. Maximal inhibition of E-selectin expression induced by TNF-α, PMA, or IL-1α requires 1- to 2-day pretreatment of HUVEC with IFN-γ (Fig. 6A). If IFN-γ was added at the same time as TNF-α, PMA, or IL-1α (Fig. 6A, time = 0), equivalent or only slightly reduced levels of E-selectin were observed when HUVEC were evaluated after 6 h (Fig. 6A). In contrast, levels of ICAM-1 were similar or slightly enhanced with IFN-γ coinubcation or 1- to 3-day pretreatment before 6-h activation with TNF-α, PMA, or IL-1α activation (Fig. 6B).

**FIGURE 4.** Effect of IFN-γ concentration on ICAM-1, HLA-DR, and activation-induced E-selectin expression. HUVEC were treated with 2.5, 25, or 250 ng/ml IFN-γ or media alone for 48 h, activated with 100 ng/ml PMA for 4 h, and then evaluated for E-selectin expression with a cell-based ELISA, as described in Materials and Methods. ICAM-1 and HLA-DR levels were measured in the absence of PMA activation. The relative expression levels of each adhesion molecule are indicated by the OD at 450 nm. The mean ± SD of triplicate samples in a single, representative experiment is shown.

**FIGURE 5.** Effect of AF-2, a neutralizing Ab to human IFN-γ, on PMA-induced E-selectin expression on HUVEC, pretreated with IFN-γ. HUVEC were cultured with 200 ng/ml IFN-γ ■ or medium alone □ for 24 h in the presence or absence of the indicated Abs at 10 μg/ml. PMA, 1 ng/ml, was then added for an additional 4 h, and cells were analyzed for expression of E-selectin by a cell-based ELISA, as described in Materials and Methods. The relative amount of E-selectin measured is indicated by the OD at 450 nm. The mean ± SD from triplicate samples in a single, representative experiment is shown. **Indicates p < 0.005; *indicates p < 0.05; NS indicates not significant, comparing with and without IFN-γ.

**Effect of IFN-γ pretreatment on activation-induced E-selectin mRNA levels in HUVEC**

To evaluate whether IFN-γ pretreatment affects E-selectin expression at the mRNA level, the levels of E-selectin mRNA in treated HUVEC were precisely measured by competitive RT-PCR (Fig. 8 (17–19). A control, the levels of ICAM-1 and GAPDH mRNA were also analyzed. cDNA was prepared from total RNA of HUVEC pretreated with IFN-γ (0 or 250 ng/ml) for 48 h and PMA (100 ng/ml) for an additional 4 h. PCR amplification of native cDNA (E-selectin, ICAM-1, or GAPDH) was performed in the presence of various known quantities of modified corresponding cDNA carrying a short deletion in the amplified region (competitor DNA) as internal standards (reviewed in Ref. 19). The PCR products from HUVEC-derived target cDNA (native PCR fragments) and those from exogenously added competitor DNA (mimic PCR fragments) were separated by agarose gel electrophoresis and quantified by gel densitometry. When the same amounts of native and mimic PCR products are produced, the concentration of competitor DNA is equal to that of target cDNA. Figure 8 (A–C) shows the ratio of native to mimic PCR products at each concentration of competitor DNA for E-selectin, ICAM-1, and GAPDH. Based on these results, it is possible to determine the difference in mRNA levels between the two cDNA samples derived from HUVEC with and without IFN-γ pretreatment. After normalization to the mRNA level of the housekeeping gene GAPDH, the level of IFN-γ mRNA was found to be slightly increased (1.6-fold) if HUVEC were first pretreated with IFN-γ. On the other hand, pretreatment with IFN-γ in PMA-activated HUVEC resulted in >fivefold reduction in the level of E-selectin mRNA (Fig. 8D).

**Effect of IFN-γ on endothelial cell expression of P-selectin**

Although PMA, TNF-α, and IL-1β induce expression of E-selectin on HUVEC, these activators do not up-regulate P-selectin expression on HUVEC (21). However, very early passage HUVEC do express a constitutive level of P-selectin that can be increased significantly by IL-4. IL-4 acts at the transcriptional level to increase P-selectin expression, and unlike E-selectin, which peaks 6 to 8 h following activator exposure, IL-4-induced P-selectin expression continues to increase and persist beyond 24 h (21). Since both E- and P-selectin have been shown to participate in neutrophil recruitment in vivo, the effects of IFN-γ on constitutive and IL-4-induced P-selectin expression on HUVEC were evaluated. Figure 9 shows that IFN-γ treatment reduces the level of IL-4-dependent P-selectin expression on HUVEC to constitutive levels. Pretreatment was not required, as maximal inhibition of IFN-γ on IL-4-induced P-selectin expression was observed when IFN-γ and IL-4 were added to cultures at the same time, for 24 h (A), or 48 h (B).
Under the conditions of these assays, a statistically significant increase in the level of cell surface P-selectin expression upon 10-min exposure to thrombin could not be detected (data not shown), in contrast to other studies (21). However, the constitutive level of cell surface P-selectin expressed by these early passage HUVEC was not reduced, but slightly increased by IFN-γ pretreatment (Fig. 9).

**Discussion**

The vascular endothelium controls the recruitment of leukocytes into tissues through the induction and modulation of leukocyte-selective endothelial cell adhesion and migration pathways. Leukocyte recruitment from the blood involves multiple steps: an initial contact or rolling step, mediated by primary adhesion receptors; chemokine or chemoattractant activation of secondary adhesion receptors; firm attachment; and transendothelial migration (22, 23). Regulation at any one of these steps can confer selectivity for a particular leukocyte subset, in that different leukocytes can preferentially utilize different adhesion molecules, or subsets of molecules at each step. For example, endothelial cell E-selectin is a strong primary adhesion receptor for neutrophils and a small subset of memory T cells, but not naive T cells or eosinophils (24–28).

The ability of cytokines to influence leukocyte-endothelial cell interactions and therefore modulate leukocyte recruitment can be a powerful mechanism through which cytokines control inflammatory and immune responses. While up-regulation of specific adhesion pathways and the subsequent recruitment of select leukocyte subsets are critically important for the inflammatory reactions that protect us from infection, it is equally important for these to be reversed once the infection is eliminated. Tight control of neutrophil recruitment is particularly required, as neutrophils are potent reservoirs of degradative enzymes and reactive oxygen species, that uncontrolled, can mediate significant tissue damage (29). Both TGF-β and IL-4 have been proposed as factors that down-regulate neutrophil recruitment in vivo, in part through their ability to inhibit the induction of E-selectin on endothelial cells (30, 31). TGF-β has general antiinflammatory properties and inhibits not only neutrophil, but also mononuclear cell adhesion to endothelial cells in vitro and recruitment in vivo (32–34). TGF-β, an important growth factor in wound healing, thus can play a major role in the resolution phase of inflammatory responses (35). IL-4 promotes
the recruitment of mononuclear cells through its ability to induce VCAM-1 on endothelial cells (36). The inhibitory effect of IL-4 on neutrophil adhesion, then, has been suggested to be important for the transition to mononuclear cell infiltration during the progression of inflammatory responses from acute to chronic (30). However, as IL-4 production is typically restricted to Th2-immune responses, the possibility that another cytokine or factor serves this role in Th1-inflammatory responses is intriguing.

Several reports have suggested that the Th1 cytokine IFN-γ has the ability to inhibit neutrophil recruitment in vivo (11–13). After observing that systemic treatment of mice with IFN-γ reduced the number of neutrophils present in thermal wounds, Amento and coworkers (11) went on to show that the number of neutrophils recruited to IL-1α-treated footpads at 4 h was reduced by 65% if mice were first systemically treated with IFN-γ. In another report, rats treated i.p. with IFN-γ shifted their response to chronic

FIGURE 8. Quantitation of E-selectin, ICAM-1, and GAPDH mRNA levels by competitive RT-PCR. A–C, HUVEC were treated with 250 ng/ml IFN-γ or medium alone □ or 48 h before 4-h activation by 100 ng/ml PMA. Following reverse transcription of total RNA, E-selectin, ICAM-1, and GAPDH cDNAs were PCR amplified using appropriate primers, in multiple aliquots with increasing twofold amounts of added E-selectin, ICAM-1, or GAPDH competitor DNAs, as described in Methods and Materials. Following separation on agarose gels, the relative amounts of PCR product derived from native cDNA or competitor DNA (mimic) were quantitated by densitometry. The x-axis shows the amount of competitor DNA, and the y-axis shows the ratio of amounts of native to mimic PCR products. The equivalence point, at which native cDNA and competitor DNA yield the same intensity RT-PCR bands, is indicated by the horizontal dotted line. The vertical dashed lines show the quantity of competitor DNA at the equivalence points. D, The relative amounts of ICAM-1 and E-selectin mRNA from PMA-activated HUVEC in IFN-γ-pretreated vs untreated HUVEC (taken from the equivalence points measured in A and B) were normalized to GAPDH levels (C), and the fold increase (or decrease) due to IFN-γ pretreatment is shown.

Pseudomonas aeruginosa infection in the lung from neutrophils to mononuclear cells, suggesting a similar effect (13). One potential mechanism contributing to these results may be related to the ability of IFN-γ to up-regulate expression of mononuclear cell chemoattractants such as monocyte-chemotactic protein-1 and IFN-γ-inducible 10-kD protein (IP-10), but not neutrophil-selective chemoattractants such as IL-8 or growth-related oncogene (GRO) (37–39). However, the known effects of IFN-γ on chemokine expression do not yet explain the inhibitory effect of IFN-γ on neutrophil recruitment, as IFN-γ is not known to down-regulate production of neutrophil-selective chemokines. On the contrary, one report describes synergy of IFN-γ with IL-1α in the induction of IL-8 by cultured endothelial cells (38).

Studies by Hallmann and coworkers (14) have suggested that an alternative mechanism by which IFN-γ can inhibit neutrophil recruitment may be through modulation of endothelial cell adhesion molecule expression or function. In these studies, neutrophil adhesion to IL-1α- or TNF-α-activated HUVEC was reduced significantly if HUVEC were first pretreated with IFN-γ. We have confirmed this observation and extended it by evaluating the activation-dependent expression of endothelial cell adhesion molecules on HUVEC with and without IFN-γ pretreatment. IFN-γ pretreatment was found to inhibit the up-regulation of E-selectin expression on HUVEC induced by TNF-α, IL-1α, or PMA. Inhibition required IFN-γ pretreatment and occurred over the same range of concentrations of IFN-γ that induce expression of HLA-DR and ICAM-1. Since both E- and P-selectin can be expressed by endothelial cells and many experimental systems have demonstrated their ability to synergize in promoting neutrophil recruitment, it was also important to evaluate the regulation of P-selectin by IFN-γ. Although HUVEC lose the ability to express P-selectin within two to three passages, primary cultures of HUVEC express a constitutive level of P-selectin that can be increased by thrombin (at 10 min) or by IL-4 (at 24–48 h) (21). Interestingly, we found that the level of P-selectin induced by 24- or 48-h activation with IL-4 was reduced significantly if HUVEC were treated with IFN-γ at the same time, while the constitutive level of cell surface P-selectin expression was slightly increased.
The regulation of endothelial cell E-selectin expression appears to be exclusively through control of mRNA production and stability (40, 41). We therefore measured the effect of IFN-γ on activation-dependent E-selectin mRNA levels. The ability of IFN-γ pretreatment of HUVEC to cause a >fivefold reduction in PMA-induced E-selectin mRNA levels suggested an effect of IFN-γ on E-selectin transcription or mRNA stability. This effect was specific, since the combination of IFN-γ pretreatment of HUVEC and PMA activation had little effect on GAPDH or ICAM-1 mRNA levels. If the effect of IFN-γ on E-selectin mRNA levels is on transcription, several hypotheses remain to be evaluated, as the E-selectin promoter contains multiple regulatory elements, including three nuclear factor-κB sites, an activating transcription factor-2 (ATF-2) site, and several high mobility group protein-1 (HMG-1) binding sites (reviewed in Ref. 40). Recently, Bennett et al. (42) proposed an interesting regulatory mechanism through which IL-4 may inhibit E-selectin expression. They suggest that the IL-4-induced transcription factor, STAT6, may act as a transcriptional repressor for E-selectin by binding to the dual nuclear factor-κB enhancer element in the E-selectin promoter. Although IL-4 is a positive regulator for P-selectin and STAT6 is not known to be induced by IFN-γ, other molecules, such as the B cell-specific BCL-6, can bind to STAT6 sequences and repress transcription (21, 43). Whether or not transcriptional repression is the mechanism through which IFN-γ inhibits activation-induced E-selectin or P-selectin expression, and whether or not the mechanism for E-selectin is similar or distinct from the mechanism for P-selectin remain to be studied. Clearly, a large variety of potential mechanisms are possible, as the molecular integration of multiple cytokine signals is an area of growing complexity.

In our experiments, adhesion of HL-60 cells to PMA-activated HUVEC was reduced ~50% if HUVEC were first preincubated with IFN-γ. While such a modest in vitro effect might not be expected to translate in vivo to a significant attenuation of neutrophil recruitment, it also must be remembered that physiologically relevant conditions in vivo are more complex. For example, in most inflammatory situations, multiple activators are acting on endothelial cells, and in many cases both E- and P-selectin participate in neutrophil recruitment (44–46). Thus, the ability of IFN-γ to inhibit activation-induced expression of both E- and P-selectin could be expected to have a more profound impact on neutrophil recruitment in vivo. It will also be important to evaluate more carefully the effect of IFN-γ on the production of leukocyte-selective chemoattractants in these more complex activating environments. The ability of IFN-γ to influence leukocyte recruitment by acting at multiple steps of the recruitment process, adhesion molecule expression, and chemoattractant production provides an attractive mechanism for tight control of a complex process by a single cytokine.

The findings described in this study can be most easily applied to situations of in vivo therapy with IFN-γ. The disparate effects of IFN-γ that have been observed in vivo may be explained, in part, by opposite effects on neutrophil vs mononuclear cell subset adhesion and recruitment. However, these results also lead to speculation on the general role of IFN-γ in inflammatory responses. In the in vitro studies presented in this work, pretreatment of HUVEC with IFN-γ was required for the inhibitory effect on E-selectin expression to be observed. The requirement for pretreatment may have been simply to overcome a time lag in the production of a (putative) IFN-γ-inducible factor responsible for inhibiting E-selectin transcription (or reducing E-selectin mRNA stability). Expression of E-selectin in vitro is quite transient, peaking at 4 to 6 h following activation, making pretreatment a requirement. In vivo, in more physiologic situations, expression of E-selectin is prolonged. E-selectin is expressed in many sites of chronic inflammation (47), and in a primate model of cerebral ischemia/reperfusion injury, E-selectin expression is still maximal at 24-h postischemia (48). In addition, pretreatment was not required for IFN-γ to inhibit IL-4-induced P-selectin expression measured at 24 h. It may be that under conditions of prolonged E- and P-selectin expression, IFN-γ can play a role in the transition of inflammatory infiltrates from neutrophil to mononuclear by modulating leukocyte adhesion and recruitment mechanisms. Evaluation of such a role for IFN-γ awaits further in vivo studies.

Although several groups have examined the effects of IFN-γ treatment of HUVEC in combination with TNF-α or IL-1β, the results that we describe have not been previously reported. In several cases, HUVEC were treated with IFN-γ at the same time as TNF-α or IL-1β, the opposite result may be due to the different culture conditions used. In addition, in our experiments we were careful to verify the activity of the IFN-γ employed, by establishing its ability to induce HLA-DR, and by utilizing blocking mAbs to IFN-γ. It is also possible that different results can be obtained with different endothelial cell donors. However, although we observe donor-dependent differences in the magnitude of IFN-γ inhibition of HUVEC E-selectin expression, HUVEC from all donors evaluated (>10) displayed significant inhibition of E-selectin induction by IFN-γ pretreatment at confluent as well as subconfluent culture conditions. The ability of IFN-γ to inhibit activation-dependent E-selectin expression also differed among activators. We found PMA-induced E-selectin up-regulation to be more sensitive to IFN-γ pretreatment than TNF-α- or IL-1β-induced expression. Interestingly, a recent report describes IFN-γ inhibition of dsRNA induced expression of E-selectin (52). The molecular mechanism for these differences remains to be defined.

Human dermal microvascular endothelial cells apparently behave differently than HUVEC in regard to E-selectin regulation. Lee and coworkers (53) report that IFN-γ induces E-selectin expression on human dermal microvascular endothelial cells, unlike HUVEC. This may be related to the observation that cutaneous inflammatory disorders, characterized by high E-selectin expression on endothelial cells, contain predominantly cutaneous lymphocyte Ag-positive memory T cells rather than neutrophils. Thus, tissue-specific differences can also modify endothelial cell responses to cytokines and other stimuli.

In conclusion, the ability of IFN-γ to control endothelial cell adhesion molecule expression and thus regulate leukocyte recruitment is a powerful mechanism through which IFN-γ can direct immune and inflammatory reactions. Novel candidate therapeutics that target components of this process (cytokines, adhesion molecules, chemokines) have a promising future for treatment of autoimmune and inflammatory conditions in which overproduction of IFN-γ has pathologic consequences.

References
MODULATION OF E- AND P-SELECTIN EXPRESSION BY IFN-γ


