Interferon-α Induces the Expression of the L-Selectin Homing Receptor in Human B Lymphoid Cells

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Abstract. The L-selectin homing receptor expressed by lymphocytes mediates the initial attachment of these cells to high endothelial venules within peripheral lymph nodes. This adhesive interaction is required for the migration of B and T lymphocytes from the blood into peripheral lymph nodes. There is currently little information regarding the nature of the factors involved in the regulation of the synthesis and expression of L-selectin by lymphocytes. In this report, the immunomodulatory cytokine interferon-α (IFN-α) was shown to markedly upregulate the surface density of L-selectin in the established human B lymphoid Daudi cell line and in a subpopulation of tissue-derived human B lymphoid cells. Other cytokines such as IFN-γ, tumor necrosis factor-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, and low molecular weight B cell growth factor did not affect L-selectin surface expression in the model Daudi B cell line. Upregulation of L-selectin surface density in IFN-α-treated Daudi B cells correlated directly with an increase in L-selectin mRNA steady state levels and enhanced L-selectin-dependent binding to a carbohydrate-based ligand, phosphomonoester core polysaccharide. Regulation of L-selectin mRNA by IFN-α had characteristics similar to that of classical IFN-stimulated genes including rapid kinetics of induction, protein-synthesis-independent induction, and sensitivity to tyrosine-kinase inhibitors. IFN-α did not upregulate L-selectin mRNA levels or surface expression in an IFN-resistant Daudi subclone which exhibits a defect in the signal transduction pathway required for the transcriptional induction of IFN-stimulated genes. These data demonstrate a fundamental role for IFN-α in regulating L-selectin synthesis and expression in human B lymphoid cells and suggest a mechanism whereby this cytokine regulates the regional trafficking of B cells to peripheral lymph nodes.

T HE generation of an active immune response is dependent on the capacity of lymphocytes to traffick continuously from the blood into secondary lymphoid organs, and back to the blood. Lymphocyte migration into secondary lymphoid tissues is initiated by the interaction of tissue-restricted homing receptors on lymphocytes with specialized structures on the luminal surface of post-capillary high endothelial venules (HEV)[1] located within peripheral lymph nodes and Peyer's patches (Gowans et al., 1964; Stamper et al., 1976; Butcher et al., 1980). Multiple lines of evidence indicate that the L-selectin homing receptor expressed on lymphocytes (also known as gp90^{Mel-14}, LAM-1, LECAM-1, Leu-8, TQ1, and DREG-56 antigen) is responsible for mediating the initial attachment of B and T lymphocytes to HEV within peripheral lymph nodes (for review see Lasky, 1992a). Monoclonal antibodies which bind L-selectin have been shown to inhibit lymphocyte adhesion to peripheral lymph node HEV, but not Peyer's patch HEV, in vitro (Gallatin et al., 1983; Kishimoto et al., 1990; Spertini et al., 1991a, b). L-selectin-specific monoclonal antibodies also inhibit in vivo localization of lymphocytes to peripheral lymph nodes without significantly affecting their trafficking to Peyer's patches (Gallatin et al., 1983; Mountz et al., 1988). Moreover, the level of expression of L-selectin on lymphocytes has been closely correlated with their ability to migrate to peripheral lymph nodes. Thus, lymphocytes which do not express L-selectin (e.g., germinal center B cells and activated T cell clones) fail to attach to peripheral lymph node HEV in vitro or home to peripheral lymph nodes in vivo (Reichert et al., 1983; Dailey et al., 1985). Finally, a definitive role for L-selectin as an organ-specific homing receptor has been established by evidence that soluble L-selectin molecules competitively inhibit lymphocyte binding to HEV in peripheral lymph nodes but not Peyer's patches (Geoffroy and Rosen, 1989). In addition to its role as a peripheral lymph node homing receptor, L-selectin has been implicated in pathological processes such as the inflam-

matory infiltration of normal tissues by lymphocytes (Spertini et al., 1991c) and metastasis of lymphoid-derived tumors to peripheral lymph nodes (Gallatin et al., 1983; Bargatze et al., 1987; Pals et al., 1991).

Differential expression of L-selectin has been observed on B lymphocyte subsets within various lymphoid compartments including the bone marrow, peripheral blood, and secondary lymphoid tissues (Kansas et al., 1985a,b; 1989; Reichert et al., 1983; Tedder et al., 1990; Salmi and Jalkanen, 1992), suggesting that the expression of this homing receptor is tightly regulated during B cell development. The regulatory mechanisms that generate B lymphocyte subsets with tissue-selective homing properties are unknown. Although it has been speculated that factors such as cytokines present in lymphoid tissue microenvironments differentially control L-selectin gene expression, to date, no cytokine has been shown to directly modulate L-selectin expression or function in B lymphocytes.

The cytokine interferon-α (IFN-α), produced primarily by activated monocytes and B cells, mediates pleiotropic biologic activities in vitro and in vivo (for reviews see Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988). In addition to its potent antiviral and antiproliferative activities, IFN-α has been shown to influence the regional trafficking of B and T lymphocytes to peripheral lymph nodes (Gresser et al., 1981; Kalaaji et al., 1988; Hein and Supersaxo, 1988; Mann et al., 1989). The hallmark of a cellular response to IFN-α is the induction of a restricted number of genes termed IFN-α-stimulated genes (ISG). Considerable insight has recently been gained into the molecular mechanisms by which IFN-α regulates ISG expression. Transcriptional stimulation results from activation of a latent transcription factor (IFN-stimulated gene factor 3, ISGF3) which recognizes a conserved *cis*-acting DNA sequence (the IFN-stimulated response element or ISRE) located within regulatory sequences of target genes (Friedman and Stark, 1985; Levy et al., 1989; Fu, 1992a; Schindler et al., 1992). The protein products encoded by ISG have been implicated in the antiviral and antiproliferative activities of IFN-α (Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988); however, a role for ISG in lymphocyte adhesion and homing has not been established.

We present here the first evidence that IFN-α regulates the expression of the L-selectin homing receptor by human B lymphoid cells. The surface density of L-selectin was markedly increased by IFN-α in the established human B lymphoid Daudi cell line and in a subpopulation of tissue-derived human B cells. In Daudi B cells, elevated L-selectin surface expression induced by IFN-α correlated directly with an increase in L-selectin mRNA steady state levels and enhanced binding to the L-selectin ligand phosphomonoester core polysaccharide (PPME) (Stoolman et al., 1987; Spertini et al., 1991a,b). The regulation of L-selectin mRNA by IFN-α had characteristics similar to that of classical ISG such as ISG15 and ISG54, including rapid kinetics of induction, protein synthesis–independent induction, and sensitivity to inhibitors of tyrosine kinase activity. IFN-α did not upregulate L-selectin mRNA levels or cell surface expression in an IFN-resistant Daudi subclone which exhibits a defect in the signal transduction pathway required for the transcriptional activation of ISG (Kessler et al., 1988). These results provide insight into the mechanisms by which the cytokine

IFN-α regulates L-selectin gene expression and further suggest that IFN-α may play a central role in directing the regional trafficking of B lymphocytes to peripheral lymphoid tissues.

Materials and Methods

Cell Isolation and Cultures

The C11-IFN growth-sensitive and the C12 IFN-resistant subclones of the human Daudi B lymphoblastoid cell line were kindly provided by Dr. A. Hovanessian (Institut Pasteur, Paris, France). C11 cells are highly sensitive to the antiproliferative effects of IFN-α with >80% growth inhibition observed following exposure to 200 IU/ml of IFN-α for 3 d whereas the C12 subclone is resistant to IFN-α at concentrations as high as 1,000 IU/ml (Hovanessian et al., 1986; Pfeffer et al., 1987; Scarozza et al., 1992). Daudi cells were maintained in logarithmic growth (5-9 × 10^5 cells/ml) in RPMI-1640 containing 10% FCS, 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10^{-5} M 2-mercaptoethanol.

Enriched populations of human tonsillar B lymphocytes were isolated essentially as described previously (Evans and Ozer, 1990; Evans et al., 1993). Cells were obtained by protocols approved by the Internal Review Board of Roswell Park Cancer Institute (Buffalo, NY). Briefly, single cell suspensions prepared from tonsil specimens obtained at the time of surgery (provided by Dr. Mark Volk, Children's Hospital of Buffalo, Buffalo, NY) were incubated 30 min at 37°C in RPMI-1640 containing 10 mg/ml carbonyl iron and iron-ingesting cells (e.g., monocytes) were removed using a magnet (Dynal, Inc., Fort Lee, NJ). Mononuclear cells (MNC) were then isolated by Ficoll-Hypaque centrifugation. Enriched populations of tonsillar B lymphocytes were obtained by rosetting with aminoethylisothiouronium-treated sheep erythrocytes for 1 h at 4°C. The E-rosette–negative population was comprised of 85–95% CD19$^+$ B cells and <10% CD3$^+$ T cells. Tonsillar lymphocyte populations were cultured in RPMI-1640 containing 2.5% FCS, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cytokines and Reagents

Recombinant human IFN-α (2.2 × 10^8 IU/mg) was kindly provided by Dr. P. Trotta (Schering Corp., Bloomfield, NJ). Recombinant human IFN-γ and IL-1β were purchased from Genzyme Corp. (Cambridge, MA). The recombinant human cytokines IL-2, IL-4, IL-6 and TNF-α were purchased from R & D Systems (Minneapolis, MN). Highly purified low molecular weight (M_r = 12,000) B cell growth factor (BCGF) was purchased from Cellular Products (Buffalo, NY). Low molecular weight BCGF is a human T cell product and is certified to be free of colony stimulating factor-1, IFN-γ, IL-1, and IL-2 activities. Cycloheximide and staurosporine were purchased from Sigma Immunochemicals (St. Louis, MO). Genistein was purchased from GIBCO-BRL (Grand Island, NY).

Monoclonal Antibodies

The following murine monoclonal antibodies specific for the human L-selectin molecule and isotype-matched control antibodies were obtained commercially: anti-Leu-8 (Becton Dickinson, Sunnyvale, CA), TQ1 (Coulter Immunology, Hialeah, FL), and DREG-56 (AMAC, Inc., Westbrook, MA). The B lymphocyte–specific monoclonal antibody anti-CD19-PE and an isotype matched PE-conjugated control antibody were purchased from Becton Dickinson.

Flow Cytometric Analysis

The cell surface expression of L-selectin was determined by direct immunofluorescence analysis as previously described (Evans et al., 1990, 1993). Briefly, cells (0.5 × 10^6 per sample) were washed in PBS/0.02% sodium azide, and then incubated with 0.5 mg/ml mouse Ig (Sigma Immunochemicals) for 10 min at 4°C to block Fc receptor sites. Cells were then incubated with the appropriately titered FITC-conjugated monoclonal anti–Leu-8 for 30 min at 4°C, washed with PBS/0.02% sodium azide, and then fixed in PBS containing 1% formaldehyde and analyzed immediately by flow cytometry. FITC-conjugated isotype matched murine antibodies that were unreactive with human leukocytes were used as negative controls. Samples were examined on a FACScan flow cytometer (Becton-Dickinson

Immunocytometry Systems, Mountain View, CA), and the data were analyzed using Consort 30 and LYSYS software (Becton-Dickinson Immunocytometry Systems). A total of 5,000 events were analyzed and specific fluorescence intensity, reported as mean channel fluorescence based on a linear scale of 0-1,000 channels, was calculated by subtracting control fluorescence values.

Two-color immunofluorescence analysis was performed to determine the effect of cytokines on L-selectin expression by tissue-derived human B lymphocytes. For these studies, enriched populations of B cells were stained simultaneously with anti-CD19-PE and anti-Leu-8-FITC immediately after their isolation from tissue or following culture for 24 h in the presence or absence of IFN-α. FITC- and PE-conjugated class-matched irrelevant control antibodies were included in all experiments and used to define appropriate quadrants for data analysis. Data from 30,000 events were collected on a FACScan flow cytometer using four-decade logarithmic amplifiers and analyzed using LYSIS software (Becton-Dickinson Immunocytometry Systems).

PPME Binding Assay

Lymphocyte binding of PPME, the phosphomonoester core from *Hansenula holstii* phosphomannan, has been described (Stoolman et al., 1987; Spertini, 1991a,b). Briefly, Daudi B cells were washed once in RPMI 1640 medium and then pretreated with the L-selectin-specific monoclonal antibody TQ1 or an isotype matched control antibody (10 μg/ml) for 30 min at 4°C. Without washing, cells were then incubated with a 1:200 dilution of fluorescein-conjugated PPME (generous gift of Dr. L. Stoolman, University of Michigan, Ann Arbor, MI) for 30 min at 4°C, and then analyzed immediately by flow cytometry. Fluorescence measurements of FITC-conjugated PPME binding were made with logarithmic amplification.

Confocal Immunofluorescence Microscopy

Daudi B cells that had been prewashed with PBS/0.02% sodium azide at 0°C were stained with the combination of FITC-conjugated anti-Leu-8 and unconjugated TQ1 monoclonal antibodies (to amplify the immunofluorescent signal) for 30 min, washed in PBS/0.02% sodium azide, and then stained an additional 30 min with goat F(ab′)₂ anti-mouse-Ig-FITC (Organon Teknika-Cappel, Malvern, PA) at a dilution of 1:30. All staining and fixing procedures were performed at 0°C in the presence of 0.02% sodium azide. Cells were then fixed 30 min in 1% formaldehyde, washed once in PBS/0.02% sodium azide, cytocentrifuged onto glass slides, and examined by confocal microscopy. Confocal laser scanning microscopy was performed using a Bio-Rad MRC-600 microscope (Bio-Rad Laboratories, Palo Alto, CA). An Argon ion laser adjusted at 488-nm wavelength was used for the analysis of fluorescein. The adjustment of the confocal system allows a field depth of ∼1 μm. The emitted signal was digitalized by Kalman filter collection and each section was scanned five times. Black and white pictures from screen images were taken on Kodak T-MAX 100 film.

L-Selectin cDNA Probe Construction

Total RNA was isolated by the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987) from IFN-sensitive Daudi B cells that had been treated with 500 IU/ml of recombinant IFN-α for 24 h. First strand cDNA was synthesized by AMV reverse transcriptase as described (Gubler and Hoffman, 1983). A 666-bp insert of the L-selectin gene was amplified using the polymerase chain reaction (PCR). L-selectin-specific primers designed from the published sequence (Tedder et al., 1989; Camerini et al., 1989) were synthesized by the Roswell Park Cancer Institute Biopolymer Facility (Buffalo, NY). The 27-mer upstream primer (GGAGAATTCGTATCTG-GAGAAGACTCTGC) contained a HindIII site and the downstream primer (CAGAAGCTTCTGAGCAGATGAAGGTAC) contained an EcoRI site. Each PCR cycle consisted of a denaturing step at 92°C for 1 min, an annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min. A total of 25 cycles were performed. The insert was subcloned into pGEM4Z (Promega, Madison, WI). The sequence of the L-selectin cDNA insert was confirmed by dideoxy sequencing.

RNA Blot Hybridization

Total RNA (30 μg/sample), isolated from IFN-α–treated and –untreated Daudi cells as described (Chomczynski and Sacchi, 1987), was denatured with glyoxal, electrophoresed on a 1.2% agarose gel, and transferred to Zetaprobe membrane (Bio-Rad Labs, Richmond, CA). The blot was then hybridized with ³²P-labeled cDNA inserts for L-selectin, ISG15, or ISG54 (cDNA probes for ISG15 and ISG54 were kindly provided by Dr. D. Levy, New York University, New York) as described (Altman et al., 1990). Inserts were labeled using the random primer technique to a specific activity of 10⁹ cpm/μg DNA. Blots were simultaneously probed with a cDNA insert for triose phosphate isomerase (TPI) (kindly provided by Dr. L. Maquat, Roswell Park Cancer Institute, Buffalo, NY) as a loading control. Washed membranes were subjected to autoradiography over a 48-h period. Scanning densitometry of the autoradiographs was performed using a Molecular Dynamics Fast Scan (Molecular Dynamics, Sunnyvale, CA).

Results

IFN-α Induces L-selectin Cell Surface Expression by Human B Lymphoid Cells

Analysis of the effect of IFN-α on the cell surface expression of L-selectin was first performed using established human B lymphoid Daudi cell lines that were either sensitive or resistant to the antiproliferative activity of IFN-α (Hovanessian et al., 1986; Pfeffer et al., 1987; Scarozza et al., 1992; Evans et al., 1993). The C11 IFN-α–sensitive and C12 IFN-α–resistant Daudi subclones express similar numbers of high affinity IFN-α receptors (Pfeffer et al., 1987); however, the IFN-resistant subclone is characterized by the failure to induce selected IFN-α–stimulated genes or ISG in response to IFN-α (Tiwari et al., 1987; Kessler et al., 1988; Wang et al., 1993). To examine the effect of IFN-α on the cell surface expression of the L-selectin peripheral lymph node homing receptor, IFN-sensitive and -resistant Daudi B cells were incubated for 24 h in medium alone or in the presence of 200 IU/ml of recombinant human IFN-α. L-selectin expression was determined by flow cytometric analysis of cells stained with the FITC-conjugated L-selectin-specific monoclonal antibody anti-Leu-8. Anti-Leu-8 binds to the NH₂-terminal extracellular lectin domain of the L-selectin molecule (Camerini et al., 1989; Tedder et al., 1990; Spertini et al., 1991b). The data shown in Fig. 1 indicate that the IFN-sensitive Daudi subclone constitutively expresses low but detectable levels of L-selectin. Incubation of IFN-sensitive Daudi cells with IFN-α for 24 h resulted in a marked increase in the cell surface density of the L-selectin homing receptor. In contrast, IFN-resistant Daudi B cells express low to nondetectable levels of L-selectin and incubation with IFN-α failed to markedly increase the cell surface density of this molecule in this cell line (Fig. 1). Similar results were obtained using two additional monoclonal antibodies, DREG-56 and TQ1 (data not shown), which also recognize epitopes within the extracellular lectin domain of L-selectin (Kishimoto et al., 1990; Spertini et al., 1991b).

Confocal scanning microscopy confirmed that IFN-sensitive Daudi B cells constitutively express low but detectable levels of L-selectin at their surface (Fig. 2). A significant increase in the cell surface density of this adhesion molecule could be detected by confocal microscopy following incubation of Daudi cells for 24 h with IFN-α. It is notable that a nonrandom distribution of L-selectin was observed on the surface of IFN-α–treated Daudi B lymphoid cells. Similar punctate immunofluorescence staining patterns of L-selectin have recently been described in neutrophils using confocal microscopy (Erlandsen et al., 1993). In this previous report, scanning electron microscopy revealed that cell surface L-selectin was localized primarily on the tips of microvilli or membrane ruffles.
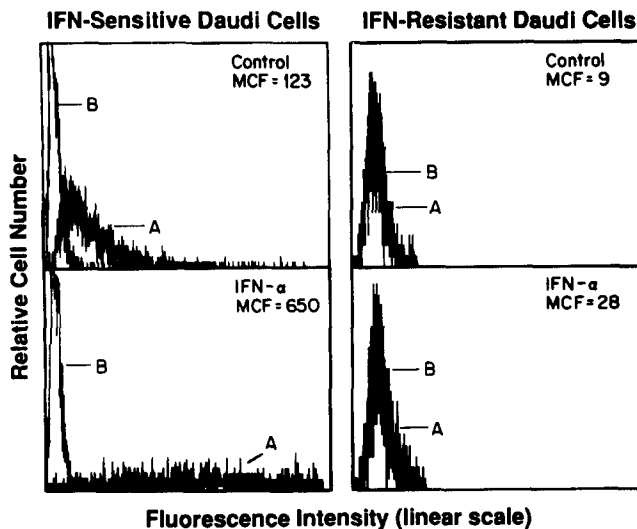
IFN-Sensitive Daudi Cells   **IFN-Resistant Daudi Cells**

Relative Cell Number

Control
MCF = 123

B

A

Control
MCF = 9

B

A

IFN-α
MCF = 650

B

A

IFN-α
MCF = 28

B

A

Fluorescence Intensity (linear scale)

Figure 1. IFN-α increases L-selectin surface expression on IFN-sensitive Daudi B lymphoid cells. IFN-sensitive and -resistant Daudi B cells were incubated 24 h in either medium alone or in the presence of 200 IU/ml of IFN-α. Cells were then stained with FITC-conjugated anti–Leu-8 monoclonal antibody (*A*) or an isotype matched FITC-labeled control antibody (*B*) and analyzed using a FACScan. The mean channel fluorescence (*MCF*) of the L-selectin signal is indicated in each panel (FITC-labeled–Ig control values have been subtracted).

The effect of IFN-α on L-selectin expression by tissue-derived human B lymphocyte populations was examined using two-color flow cytometric analysis. In general it has been difficult to evaluate the effect of cytokines on L-selectin expression by primary human lymphocytes since culture of these lymphocyte populations in medium alone, in the absence of exogenous cytokines, causes a marked increase in L-selectin expression (Tedder et al., 1990; Salmi and Jalkanen, 1992; S. S. Evans, unpublished observations). The molecular basis for this culture-associated increase in L-selectin expression is not understood. In the present study, lymphocytes were cultured in medium containing low concentrations of FCS (i.e., 2.5%) to minimize the level of spontaneous increase in L-selectin expression as described (Salmi and Jalkanen, 1992). It should be noted, however, that in some experiments reduction of FCS concentrations

did not prevent the high level of culture-associated increase in L-selectin expression; the effects of IFN-α on L-selectin expression were not evaluated under these conditions.

In the experiment shown in Fig. 3, a majority of B lymphocytes isolated from human tonsil were found to be L-selectin negative. Following incubation for 24 h with IFN-α, 20% of tonsillar B cells became L-selectin positive while only 9% expressed L-selectin after culture in medium alone (Fig. 3). A similar increase in L-selectin expression by a small subpopulation of tonsillar B cells was observed in multiple experiments involving specimens from different individuals. Moreover, ~10–15% of B cells within human bone marrow, spleen, and peripheral blood were found to increase L-selectin expression in response to IFN-α (data not shown). Although the proportion of tissue-derived B cells responding to IFN-α appeared to be relatively restricted, it is of note that the L-selectin density on IFN-α–responsive Daudi-B cells and tissue-derived B cells was comparable with that normally found on blood lymphocytes which are functionally capable of trafficking to peripheral lymph nodes in vivo. Because of the relatively uniform increase in L-selectin expression by Daudi B cells in response to IFN-α, this cell line was used as a model in subsequent studies to investigate further IFN-α regulation of L-selectin expression.

Several other cytokines are known to influence the expression of adhesion molecules on diverse cell types. For example, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) have been demonstrated to increase the expression of the vascular selectin ELAM-1 (or E-selectin) as well as the vascular cell adhesion molecule-1 (VCAM-1) by human umbilical chord endothelial cells (Osborn et al., 1989). TNF-α, IL-1β, and IFN-γ have also been shown to induce intercellular adhesion molecule-1 (ICAM-1) cell surface expression and mRNA levels in cells of multiple lineages (Pober et al., 1986; Simmons et al., 1988). Therefore, the effect of these and other cytokines on L-selectin expression by IFN-sensitive Daudi B cells was examined. Daudi cells were incubated 24 and 48 h with biologically active concentrations of IFN-α, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, and low molecular weight B cell growth factor (BCGF) and the relative expression of L-selectin was determined by flow cytometry (Fig. 4). Again, IFN-α significantly upregulated L-selectin expression as indicated by a major shift in specific fluorescence intensity of cells stained with FITC-conjugated
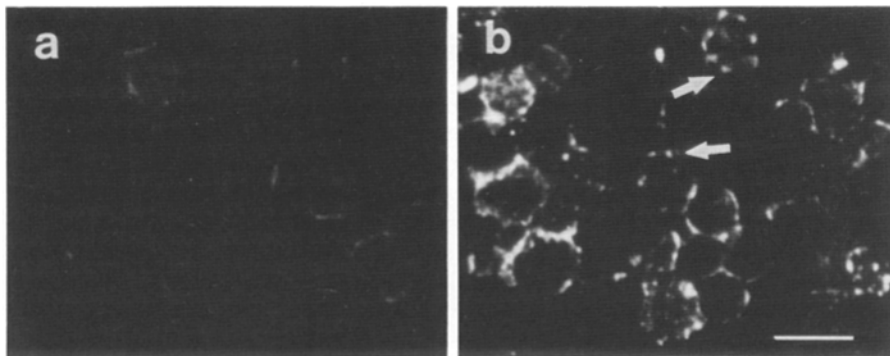

Figure 2. Confocal immunofluorescence microscopy of Daudi B lymphoid cells. IFN-sensitive Daudi cells were incubated 24 h in medium alone (*a*) or in the presence of 500 IU/ml of IFN-α (*b*) and then stained with murine monoclonal antibodies specific for L-selectin (FITC-conjugated anti–Leu-8 and TQ1) in combination with goat F(ab′)₂ anti–mouse-IgG-FITC. All staining and fixation procedures were performed at 0°C in the presence of 0.02% sodium azide. Cells were subsequently fixed in 1% formaldehyde and then cytocentrifuged onto glass slides and examined by confocal microscopy. Arrows indicate punctate surface staining of L-selectin. Immunofluorescence was not detected using a FITC-conjugated isotype matched control antibody or goat F(ab′)₂ anti–mouse-IgG-FITC secondary antibodies (not shown). Bar, 25 μm.
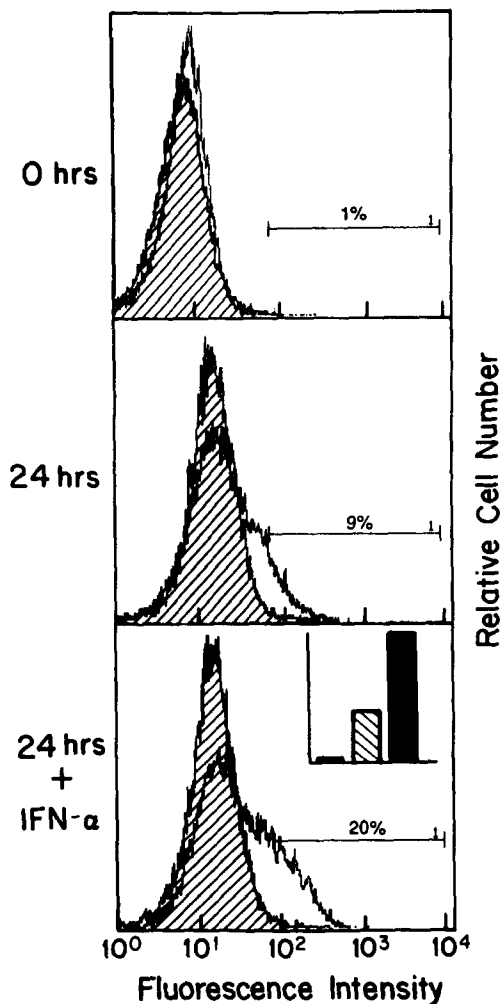
Figure 3. IFN-α increases L-selectin surface expression by a subpopulation of human tonsillar B lymphocytes. Enriched tonsillar B lymphocyte populations (>90% CD19⁺) were stained simultaneously with the B cell-specific monoclonal antibody anti-CD-19-PE and the L-selectin-specific monoclonal antibody anti-Leu-8-FITC either immediately after isolation from tissue or following culture for 24 h in the absence or presence of 500 IU/ml of IFN-α. Total B cell recovery was not affected by culture with IFN-α. L-selectin expression is depicted only for the CD19⁺ B cell population. The hatched histograms represent the background fluorescence of cells stained with a FITC-labeled isotype matched negative control antibody. The frequency of L-selectin–positive B cells (designated by region 1) is shown. The relative number of L-selectin–positive B cells at the time of isolation from tissue (□) or following culture in the absence (⊠) or presence (■) of IFN-α is indicated in the inset. Results are representative of four independent experiments.

anti-Leu-8. On the other hand, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, and low molecular weight BCGF did not markedly alter the cell surface density of L-selectin on Daudi B lymphoid cells over a 48-h period. These results are consistent with recent studies indicating that physiologic concentrations of IL-1, IL-3, IL-4, IL-5, IL-6, IFN-γ, and TNF fail to significantly alter L-selectin cell surface expression by human lymphocytes derived from the bone marrow or peripheral blood (Tedder et al., 1990; Salmi and Jalkanen, 1992; Picker et al., 1993).

IFN-α was also found to induce L-selectin expression by Daudi B cells in a time- and dose-dependent manner. A significant increase in the cell surface density of L-selectin could be detected 9 h after the addition of IFN-α and by 24 h, L-selectin expression was increased approximately ten-fold above background levels (Fig. 5). Moreover, elevated L-selectin cell surface expression was maintained during continuous exposure to IFN-α for 72 h. In untreated Daudi cells, the basal level of L-selectin expression decreased over a 72-h period as cells approached confluence and G_0/G_1 growth arrest (Scarozza et al., 1992). Previous studies have similarly noted an inverse relationship between L-selectin cell surface expression by cell lines and cell culture concentration (Tedder et al., 1990). Our studies further indicate that maximal induction of L-selectin occurred at physiologic concentrations of IFN-α (i.e., ≤100 IU/ml) and was inhibited by the protein synthesis inhibitor cycloheximide (Fig. 6). L-selectin induction was also abrogated by the broad spectrum protein kinase inhibitor staurosporine and by the tyrosine kinase-specific inhibitor genistein (Fig. 6). These protein synthesis and protein kinase inhibitors also down-regulated the basal expression of L-selectin at the cell surface of untreated Daudi cells, suggesting that turnover of the L-selectin molecule in the plasma membrane occurs continuously over a 24-h period.

IFN-α Increases the Steady-state Level of L-selectin mRNA

There are several possible mechanisms which could account for the increased cell surface expression of L-selectin observed in IFN-treated Daudi cells. Since L-selectin is known to be continuously shed from the cell surface of lymphocytes (Jung and Dailey, 1990; Schleiffenbaum et al., 1992), upregulation of L-selectin could occur as a result of stabilization of L-selectin in the plasma membrane. Alternatively, IFN-α may regulate L-selectin synthesis in Daudi B lymphoid cells, as suggested by evidence that cycloheximide inhibits the IFN-α–induced increase in L-selectin cell surface expression. This question was addressed by examining the effect of IFN-α on L-selectin mRNA accumulation in IFN-sensitive and -resistant Daudi cells. Northern blot analysis of total RNA isolated from untreated IFN-sensitive C11 Daudi cells indicated that these cells contain detectable levels of the 2.4-kb L-selectin mRNA transcript (Fig. 7 a), consistent with the flow cytometric data shown in Fig. 1. Following incubation with IFN-α, increased steady state levels of L-selectin mRNA were detectable within 3 h, reached maximum levels by 12 h, and remained elevated over a 72-h period during continuous exposure to IFN-α. Scanning densitometry of the autoradiograph revealed that L-selectin mRNA levels increase approximately 14-fold after exposure to IFN-α for 12 h (Fig. 7 b). In contrast, the IFN-resistant C12 Daudi subclone, which express low to nondetectable levels of L-selectin at the cell surface (Fig. 1), contained negligible levels of L-selectin mRNA in both the untreated and 24-h IFN-α–treated samples (Fig. 7). In addition, other cytokines such as TNF-α and IFN-γ had relatively little effect on L-selectin mRNA levels over a 48-h period in IFN-sensitive or -resistant Daudi cells (data not shown). Taken together, these data support the conclusion that IFN-α induction of L-selectin surface expression results from increased synthesis of this homing receptor.
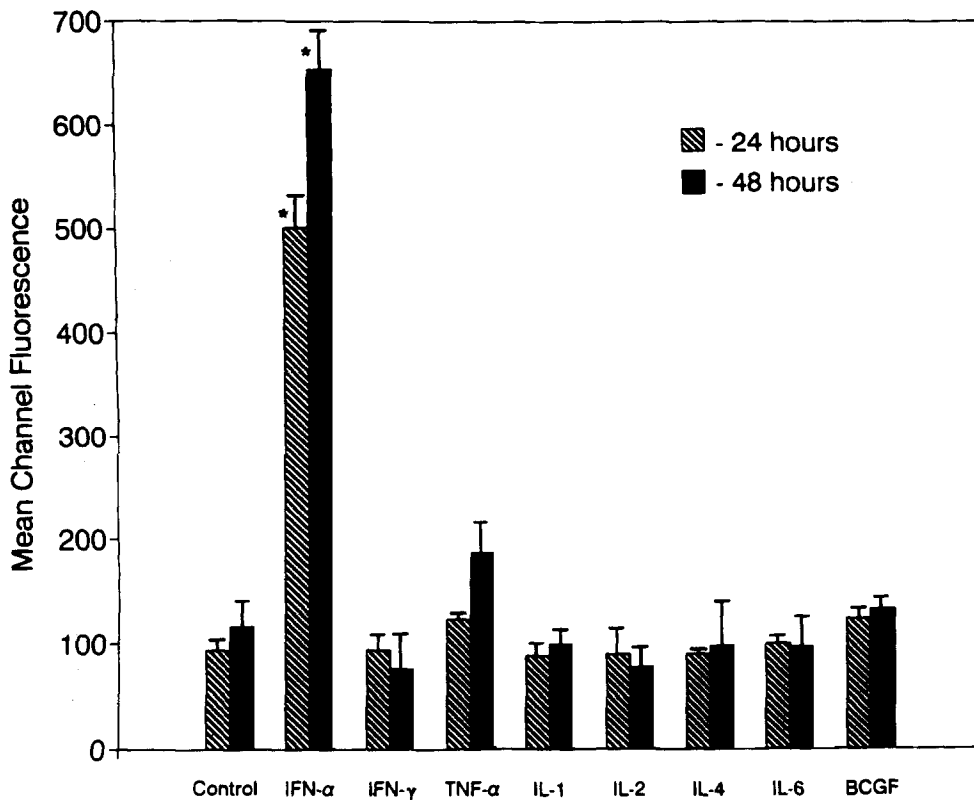
Figure 4. L-selectin surface expression is selectively induced by the cytokine IFN-α. IFN-sensitive Daudi B cells were incubated 24 or 48 h in medium alone or medium supplemented to a final concentration of 500 IU/ml of IFN-α, 500 IU/ml of IFN-γ, 100 ng/ml of TNF-α, 10 IU/ml of IL-1β, 100 IU/ml of IL-2, 5 ng/ml of IL-4, 5 ng/ml of IL-6, or 10% (vol/vol) low molecular weight BCGF. Cells were then stained with FITC-conjugated anti–Leu-8 monoclonal antibody and the relative density of L-selectin on Daudi B cells was determined by flow cytometric analysis. Values represent mean ± SD of three independent experiments. Differences between control cells and IFN-α–treated cells were highly significant (*P < 0.0002 using the t-test).
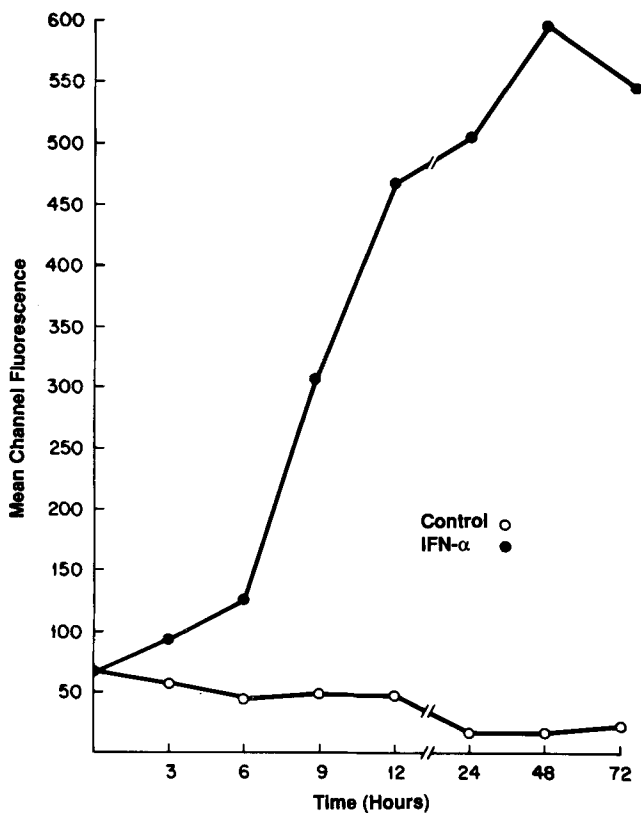

Figure 5. Kinetics of L-selectin cell surface induction by IFN-α. IFN-sensitive Daudi B cells, plated at an initial concentration of

Two important features have been identified regarding the signal transduction pathway utilized by IFN-α to transcriptionally induce ISG expression. Transcriptional activation of ISG has been shown to occur in the absence of ongoing protein synthesis and to be dependent on tyrosine kinase activity (Levy et al., 1988; Schindler et al., 1992; Fu, 1992a; David et al., 1993; Wang et al., 1993). Therefore, to determine if this IFN-α signal transduction pathway could play a role in the regulation of L-selectin gene expression, we examined the effects of cycloheximide and genistein on IFN-α induction of the mRNA steady state levels of L-selectin and two well-characterized IFN-α–inducible genes, ISG15 and ISG54, which encode 15- and 54-kD cytoplasmic proteins, respectively (Levy et al., 1988, 1989; Kessler et al., 1988). The data shown in Fig. 8 indicate that the mRNA levels of ISG15, ISG54, and L-selectin were markedly induced following incubation for 4 or 8 h with IFN-α. Moreover, upregulation by IFN-α of ISG15, ISG54, and L-selectin mRNA levels occurred in the presence of cycloheximide but was significantly reduced in genistein-treated cells. Taken together with evidence that IFN-α did not induce L-selectin mRNA levels in the IFN-resistant Daudi subclone characterized by a defect in the transcriptional induction of IFN-α–stimulated genes (ISG) (Tiwari et al., 1987; Kessler et al.,

6 × 10⁵ cells/ml, were incubated in medium alone (○) or with 200 IU/ml of IFN-α (●) for the indicated time intervals. Cells were then stained with FITC-conjugated anti–Leu-8 monoclonal antibody and L-selectin expression was assessed by flow cytometry.
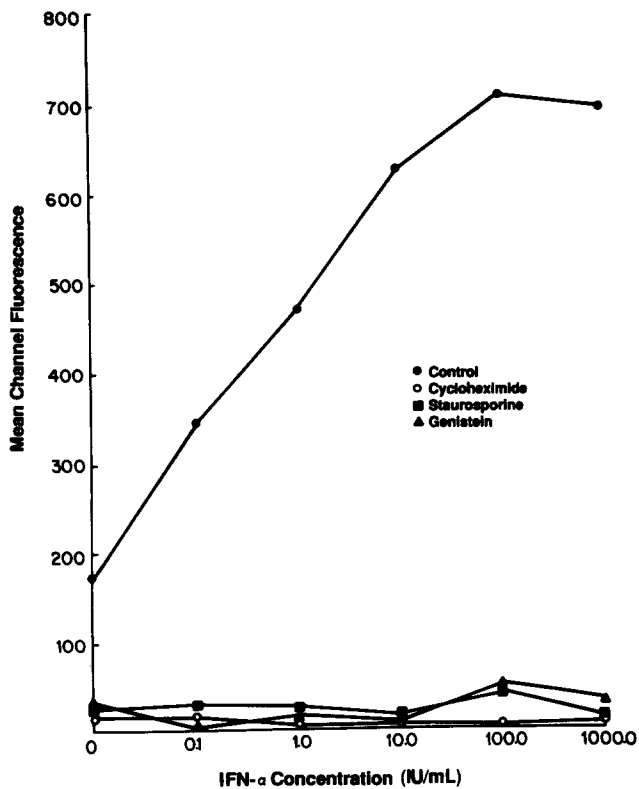
Figure 6. L-selectin induction by IFN-α is blocked by inhibitors of protein synthesis and of protein kinase activity. IFN-sensitive Daudi B cells were pre-incubated 30 min in medium alone (●) or in the presence of 50 μM cycloheximide (○), 500 nM staurosporine (■), or 185 μM genistein (▲) before the addition of IFN-α at 200 IU/ml. Following culture for an additional 24 h, cells were stained with the FITC-conjugated anti–Leu-8 monoclonal antibody and analyzed by flow cytometry. Cell viability was >85% under these conditions.
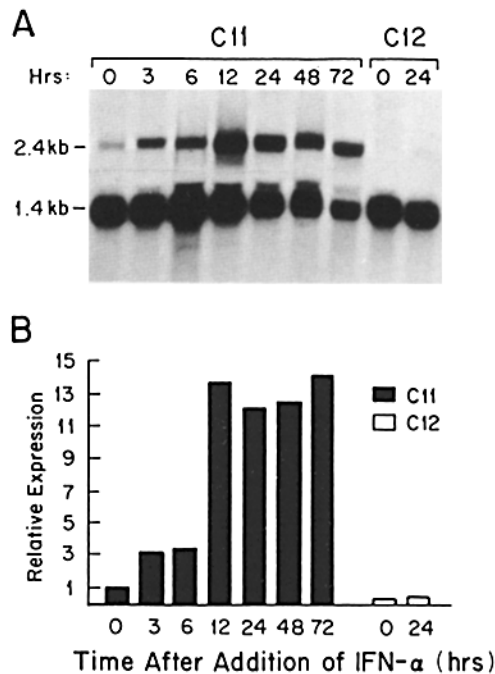

Figure 7. Northern blot analysis of the expression of L-selectin mRNA in Daudi B lymphoid cells. (A) IFN-sensitive C11 and -resistant C12 Daudi B cells were incubated with 500 IU/ml of recombinant human IFN-α for the indicated time intervals, harvested, and total cellular RNA was prepared. 30 μg of each sample were electrophoresed through an agarose gel and transferred to a Zetaprobe membrane and the blot was hybridized with radiolabeled L-selectin and triose phosphate isomerase (TPI) cDNA inserts. The 2.4 kb L-selectin transcript and the 1.4-kb TPI transcript are indicated. (B) The same RNA blot was quantified by densitometric scanning and normalized with respect to TPI mRNA to correct for small variations in the amount of input RNA. The relative expression of L-selectin mRNA was calculated based on L-selectin mRNA levels detected in untreated IFN-sensitive C11 cells.

1988; Wang et al., 1993), these data strongly suggest that the regulation of classical ISG mRNA and L-selectin mRNA levels by IFN-α involves common signalling pathways.

IFN-α Augments L-selectin–mediated Binding to the Carbohydrate Substrate PPME

L-selectin binds via its NH2-terminal lectin domain to carbohydrate-rich ligands on HEV (Imai et al., 1991; Lasky et al., 1992b) and this interaction can be inhibited by specific polysaccharides rich in mannose 6-phosphate such as the phosphomonoester core polysaccharide (PPME) derived from yeast (Stoolman et al., 1987). PPME has also been conjugated with fluorescein to assess L-selectin–binding activity without interference from other adhesion pathways (Spertini et al., 1991a,b). Therefore, to determine if the L-selectin molecules induced by IFN-α were functionally capable of binding oligosaccharide ligands, flow cytometry was used to assess PPME-FITC binding to Daudi B lymphoid cells (Fig. 9). The specificity of PPME binding to L-selectin was confirmed by the ability of the L-selectin–specific monoclonal antibody TQ1 to inhibit PPME binding to cells. In the absence of IFN-α, PPME-FITC binding to the IFN-sensitive Daudi subclone could not be detected despite the expression of low levels of cell surface L-selectin, suggesting that a

threshold level of L-selectin is necessary to measure PPME binding by flow cytometry. Incubation of IFN-sensitive Daudi B lymphoid cells with IFN-α resulted in the marked enhancement of L-selectin–mediated binding to PPME which correlated directly with the increased cell surface density of L-selectin in these cells. In contrast, IFN-α did not enhance binding of the FITC-conjugated PPME substrate by the IFN-resistant Daudi subclone.

Discussion

In this report we provide the first evidence of a role for a specific cytokine, IFN-α, in regulating the expression of the L-selectin peripheral lymph node homing receptor by human B lymphoid cells. IFN-α was shown to induce a five to 10-fold increase in the cell surface density of L-selectin in the IFN-α sensitive Daudi B lymphoid cell line. A marked increase in L-selectin density in response to IFN-α was also observed in subpopulations of B cells isolated from human tonsil, spleen, peripheral blood, and bone marrow. The density of L-selectin expressed on IFN-α–responsive Daudi B cells and tissue-derived B cells was comparable with that found on human lymphocytes which are functionally capable
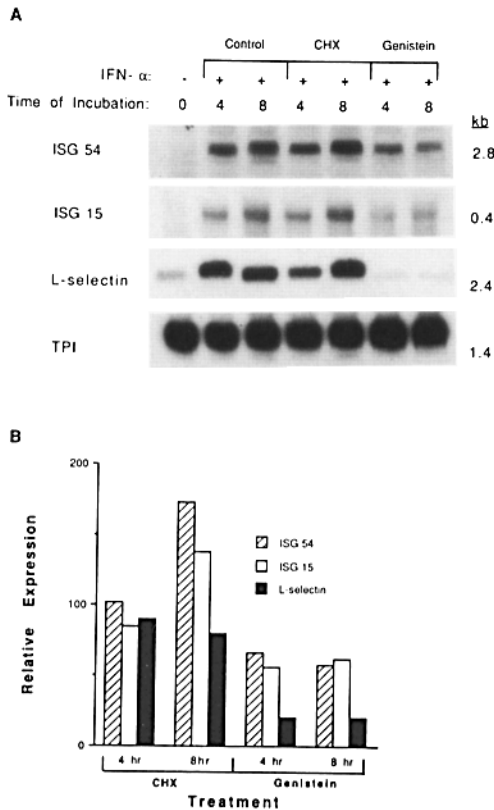
A

B

Figure 8. Effect of cycloheximide and genistein on L-selectin and ISG expression induced by IFN-α treatment of IFN-sensitive Daudi cells. (A) IFN-sensitive Daudi B cells were pre-incubated 30 min in medium alone or in the presence of 50 μM cycloheximide (CHX) or 185 μM genistein before the addition of 500 IU/ml of IFN-α. After incubation for 4 or 8 h, total RNA was isolated and 30 μg of each sample were electrophoresed through an agarose gel and transferred to a Zetaprobe membrane. The blot was hybridized with radiolabeled ISG54, ISG15, L-selectin, and TPI cDNA inserts. The ISG54 (2.8 kb), ISG15 (0.4 kb), L-selectin (2.4 kb), and TPI (1.4 kb) transcripts are indicated. (B) The RNA blots were quantified by densitometric scanning and normalized with respect to TPI mRNA to correct for small variations in the amount of input RNA. The relative expression of ISG54, ISG15, and L-selectin mRNA was calculated based on the level of expression of these genes in IFN-α-treated Daudi B cells.

of trafficking to peripheral lymph nodes. It is of interest that the response to IFN-α was restricted to approximately 10–15% of primary human B lymphocyte populations. These results may reflect a differential response to IFN-α by tissue-derived B cells which are highly heterogeneous with respect to stage of maturation, differentiation, and activation. The relatively uniform increase in L-selectin expression by Daudi B cells in response to IFN-α suggests that this established cell line could serve as valuable model in which to examine the molecular mechanisms by which L-selectin expression and synthesis are regulated.

Upregulation of L-selectin cell surface expression by IFN-sensitive Daudi cells in response to IFN-α was shown to be sensitive to cycloheximide and was temporally correlated with an increase in the steady-state levels of L-selectin mRNA. These data are consistent with the conclusion that IFN-α regulates the synthesis of this homing receptor. Con-
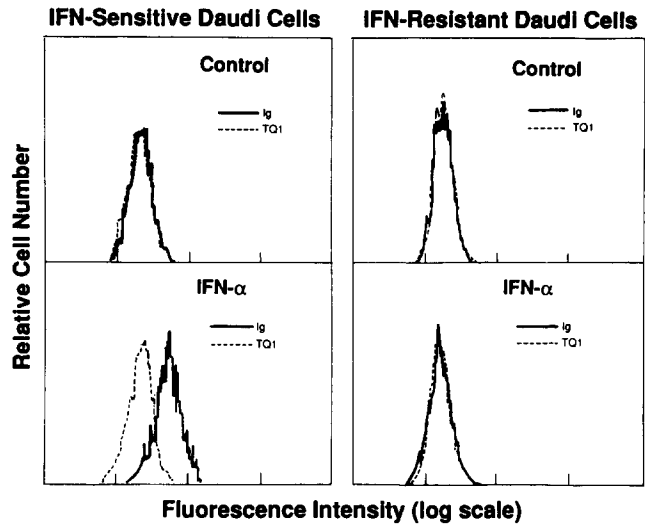

Figure 9. IFN-α increases L-selectin–mediated binding of FITC-conjugated PPME to IFN-sensitive Daudi B Lymphoid cells. IFN-sensitive and IFN-resistant Daudi cells were incubated 24 h in the absence (control) or presence of 500 IU/ml of IFN-α. Cells were then incubated with either the L-selectin–specific monoclonal antibody TQ1 (--) or an isotype matched control Ig (—) before the addition of FITC-conjugated PPME and fluorescence intensity of cell staining was analyzed by flow cytometry.

siderable insight has recently been gained into the signal transduction pathway utilized by IFN-α to transcriptionally activate ISG and it is possible that these mechanisms play a role in controlling L-selectin gene expression in human B lymphoid cells. Receptor occupancy by IFN-α has been shown to rapidly trigger the activation of a transcriptional factor termed ISGF3α (interferon-stimulated gene factor 3α) which is comprised of three cytoplasmic proteins of 113, 91, and 84 kD (Fu et al., 1992b). ISGF3α activation involves phosphorylation of tyrosine residues on all three of its subunits and their association with a 48-kD DNA-binding protein termed ISGF3γ (Levy et al., 1989; Fu, 1992a; Schindler et al., 1992; Fu, 1992a; David et al., 1993). Following activation, this multimeric ISGF3 complex translocates to the nucleus where it elicits a transcriptional response by binding with high affinity to specific IFN-stimulated response elements (ISRE) located in the promoter region of inducible genes such as ISG15, ISG54, and the 6-16 gene (which encode proteins of 15, 54, and 12 kD, respectively) (Friedman and Stark, 1985; Levy et al., 1988).

The mechanism by which IFN-α increases the accumulation of L-selectin mRNA levels in Daudi cells was not directly examined in the present study. Thus, upregulation could occur as a result of transcriptional activation of the L-selectin gene or increased stability of L-selectin mRNA. However, several notable similarities exist between the regulation by IFN-α of well characterized ISG (i.e., ISG15, ISG54) and L-selectin gene expression, suggesting that induction of these genes involves common signal transduction pathways. Specifically, the kinetics of L-selectin mRNA induction by IFN-α parallels the time course of induction of mRNAs encoded by ISG15, ISG54 and the 6-16 gene in the IFN-sensitive Daudi B cell line (Tiwari et al., 1987; Kessler et al., 1988; Wang et al., 1993). Induction of the L-selectin

mRNA, and ISG15, ISG54, and the 6–16 gene occurs within 3 h following exposure to IFN-α and elevated levels of these genes are maintained for periods greater than 12 h in the IFN-sensitive Daudi B lymphoid cell line. It is of note that IFN-α–induced upregulation of L-selectin mRNA preceded the characteristic IFN-α–mediated G_0/G_1 arrest that occurs over a 24–48-h period in Daudi cells (Einat et al., 1985; Scarozza et al., 1992), indicating that modulation of this homing receptor gene cannot be attributed to an overall change in cell cycle distribution. Our data further indicate that induction by IFN-α of L-selectin, ISG15, and ISG54 mRNA steady state levels in Daudi cells is dependent on tyrosine kinase activity but does not require ongoing protein synthesis, two hallmarks of the signal transduction pathway utilized by IFN-α to transcriptionally activate ISG. Finally, IFN-α failed to induce L-selectin mRNA levels or cell surface expression in the IFN-resistant Daudi subclone. This IFN-resistant Daudi subclone is characterized by abnormally low levels of nuclear ISGF3 activity following IFN-α binding to cell surface receptors and low to non-detectable levels of ISG15, ISG54, and the IFN-inducible mRNA 561 (Tiwari et al., 1987; Kessler et al., 1988; Wang et al., 1993). Taken together, these data are consistent with a model in which regulation of L-selectin gene expression by IFN-α involves direct activation by the ISGF3 transcriptional factor. To date, the promoter region and transcription initiation site of the L-selectin gene have not been identified (Ord et al., 1990) and therefore it remains to be determined if ISRE consensus sequences are present in the regulatory region of the L-selectin gene.

The increase in the cell surface density of L-selectin on human B lymphoid cells in response to IFN-α was also shown to correlate with an increase in L-selectin–mediated binding to the mannose-6-phosphate–rich carbohydrate ligand PPME. Previous studies have established that the level of PPME binding directly correlates with the ability of lymphocytes to adhere to peripheral lymph node HEV (Stoolman et al., 1987; Spertini et al., 1991a,b). Thus, upregulation of L-selectin by IFN-α would be expected to enhance the entry of lymphocytes into peripheral lymph nodes via HEV. Evidence that IFN-α affects lymphocyte entry into peripheral lymph nodes has been described in sheep and rodent models. In these studies, systemic administration of IFN-α over periods greater than 6 h was shown to result in a decrease in the total number of B and T lymphocytes in the circulation and an associated increase in both the uptake and retention of lymphocytes within peripheral lymph nodes (Gresser et al., 1981; Kalaaji et al., 1988; Hein and Supersaxo, 1988; Mann et al., 1989). Moreover, in short-term homing studies, preincubation of rodent lymphocytes with IFN-α/β prior to intravenous transfer into syngeneic hosts increased their rate of entry into peripheral lymph nodes without affecting their localization in Peyer's patches (Kimber et al., 1987). In contrast, enhanced migration of nontreated lymphocytes to peripheral lymph nodes was not observed when these cells were injected into IFN-α–treated mice (Gresser et al., 1981). These results, taken together with the data of the present report, support the hypothesis that IFN-α influences the regional trafficking of lymphocytes to peripheral lymph nodes, in part, by acting at the lymphocyte level to regulate L-selectin expression.

It remains to be determined if IFN-α can account for the differential regulation of L-selectin expression in B cell subsets observed in vivo. Within the bone marrow, immature B cells ($CD10^+CD20^-CD19^+$) have been shown to be L-selectin negative while more mature B cells ($CD10^-CD20^+CD19^+$) reportedly express high levels of this homing receptor (Kansas and Dailey, 1989; Salmi and Jalkanen, 1992). In the peripheral blood, approximately 80% of peripheral blood B cells express high levels of L-selectin and naive B cells have been identified within this population (Kansas et al., 1985b; Tedder et al., 1990). The numerically minor L-selectin–negative peripheral blood B cell population has been shown to be functionally more mature as determined by the production of antibodies in response to helper T cells or mitogens (Kansas et al., 1985b). In secondary lymphoid tissues (e.g., lymph nodes, tonsil, Peyer's patches), germinal center B cells have been also shown to express low to non-detectable levels of L-selectin (Reichert et al., 1983; Kansas et al., 1985a). B cells which traffick through peripheral lymphoid tissues have been proposed to continuously alter their expression of tissue-restricted homing receptors during their conversion from sessile to migratory cells or as they enter the circulating memory pool. Based on evidence obtained in the present study, it is tempting to speculate that IFN-α, present in lymphoid tissue microenvironments, differentially regulates L-selectin receptor expression on human B lymphocytes. There is little information available regarding the physiologic concentration of cytokines including IFN-α in B lymphoid compartments in vivo, in part because of the difficulty of assessing the concentration of cytokines in local environments within cell-to-cell contact regions. Future studies are expected to provide further insight into the molecular mechanisms by which IFN-α regulates L-selectin gene expression by B lymphocytes and the physiologic role of IFN-α in controlling the homing behavior of human B lymphocytes in vivo.

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