Monocyte Rolling, Arrest and Spreading on IL-4-activated Vascular Endothelium under Flow Is Mediated via Sequential Action of L-Selectin, \(\beta_1\)-Integrins, and \(\beta_2\)-Integrins

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Abstract. Leukocyte interactions with vascular endothelium at sites of inflammation can be dynamically regulated by activation-dependent adhesion molecules. Current models, primarily based on studies with polymorphonuclear leukocytes, suggest the involvement of multiple members of the selectin, integrin, and immunoglobulin gene families, sequentially, in the process of initial attachment (rolling), stable adhesion (arrest), spreading and ultimate diapedesis. In the current study, IL-4-activated human umbilical vein endothelium, which selectively expresses VCAM-1 and an L-selectin ligand but not E-selectin, and appropriate function blocking monoclonal antibodies, were used to study monocyte-endothelial interactions in an in vitro model that mimics microcirculatory flow conditions. In this system, L-selectin mediates monocyte rolling and also facilitates \(\alpha_\beta_1\)-integrin-dependent arrest, whereas \(\beta_2\)-integrins are required for spreading of firmly attached monocytes on the endothelial cell surface but not their arrest. These findings provide the first in vitro evidence for human monocyte rolling on cytokine-activated endothelium, and suggest a sequential requirement for both \(\beta_1\)- and \(\beta_2\)-integrin-dependent adhesive mechanisms in monocyte-endothelial interactions.

Peripheral blood monocytes interact with the vascular endothelial lining as an initial step in a wide range of pathological processes including acute and chronic inflammation, immune reactions, and atherosclerosis (12, 13, 45). As a consequence of their transendothelial migration, monocytes are recruited into tissues, organs and body cavities, undergo maturation to macrophages, and participate in defending the host against invading pathogens and regulating the behavior of vascular and non-vascular cells through the secretion of cytokines and other chemical mediators.

Early in vitro studies of monocyte adhesion to cultured endothelial cells were typically performed under static conditions and indicated that basal adhesion of purified blood monocytes was relatively high (2, 5, 6, 9, 15, 20, 21, 38, 40) compared with neutrophils or lymphocytes (9, 34, 35, 38, 63). Activation of the endothelium with TNF-\(\alpha\), IL-1, and LPS (6, 20), resulted in a 2–5-fold increase in monocyte adhesion. This enhanced monocyte adhesion reflects the induction of multiple adhesion molecules on the endothelial surface including E-selectin, which interacts with sialyl-Lewis\(^{x}\) and similar carbohydrate ligands; Vascular Cell Adhesion Molecule-1 (VCAM-1), which interacts with \(\alpha_\beta_1\)-integrins; and Intercellular Adhesion Molecule-1 (ICAM-1), which interacts with \(\beta_2\)-integrins (CD11a/CD18 and CD11b/CD18) (4, 9, 20, 57). That multiple molecules are induced suggests redundancy or overlap in their function. However, recent studies performed in vivo, or in vitro under defined flow conditions, have further clarified the conceptual framework of leukocyte-endothelial adhesion by revealing that various receptor-ligand pairs can function in a sequential manner to mediate these adhesive interactions (7, 62). In the molecular models that have been proposed, the initial attachment of the blood leukocyte is mediated, in part, by L-selectin.
tin, while subsequent firm adhesion (arrest) is mediated by β2-integrins. However, these models are primarily based on observations with polymorphonuclear leukocytes (in particular neutrophils), and the extent to which monocytes conform to this paradigm remains untested. In addition to expressing both L-selectin and β2-integrins, blood monocytes also express multiple β1-integrins (53), of which the αvβ1 molecule has been shown to interact with both matrix proteins and the endothelial expressed molecule, VCAM-1 (16, 58). Thus, although αvβ1, interacting with its endothelial ligand VCAM-1, has been implicated in monocyte recruitment during early atherosclerotic plaque formation in vivo (11–13), and in monocyte adhesion to activated endothelial cells in vitro (8, 9, 20, 41, 55), its potential role in monocyte adhesion under defined flow conditions has yet to be examined.

Interleukin-4 (IL-4), originally described as a costimulatory factor necessary for B-cell proliferation (23, 66), has been shown to exert pleiotropic effects on several blood cell types in vitro including B cells, activated T cells, thymocytes, mast cells, and monocytes/macrophages (for review see 66). Recent in vitro studies under static conditions have demonstrated that IL-4 also can stimulate cultured vascular endothelium to increase its adhesiveness for peripheral blood lymphocytes (36, 61), eosinophils and basophils, but not neutrophils (49), and to induce the synthesis of monocyte chemotactic peptide-1 (MCP-1) (42, 44), a monocyte-directed chemoattractant (37, 43, 67). In contrast to the cytokines that are known to cause the sequestration of various leukocytes, IL-4 selectively induces surface expression of VCAM-1, does not alter ICAM-1 expression and does not induce E-selectin expression (36, 49, 60, 61).

The aim of the present study was to examine in detail the cellular processes and molecular mechanisms of monocyte-endothelial adhesion using an in vitro flow model (50). This system allows direct microscopic examination of monocyte-endothelial interactions, live-time, under a range of defined laminar flow conditions that mimic blood flow in post-capillary venules (22). We used IL-4 activation of cultured human umbilical vein endothelial cell (HUVEC) which selectively induces both VCAM-1 and an L-selectin ligand but not E-selectin (36, 49, 60, 61), and appropriate function-blocking mAb, to investigate the molecular mechanisms involved in monocyte-endothelial adhesive interactions under defined flow conditions. The results demonstrate that L-selectin mediates monocyte rolling and facilitates arrest through αvβ1-integrins interacting with VCAM-1. In contrast β2-integrins were required for the spreading of firmly attached monocytes on the endothelial surface, but not for their arrest. These data thus suggest a complex, sequential, molecular model for monocyte-endothelial adhesive interactions under defined flow conditions.

**Materials and Methods**

**Materials**

EDTA and Hepes were purchased from Sigma Chem. Co. (St. Louis, MO). Human serum albumin (HSA, Buminate 25%, sterile and nonglycrogenic solution) was obtained from Baxter Healthcare Corp. (Glendale, CA). HBSS with or without Ca2+ and Mg2+, DPBS, M199, DMEM for hybridoma culture and RPMI1640 with 25 mM Hepes were purchased from Whittaker Bioproducts (Walkersville, MD). rH-IL-4 (produced in E. coli) was obtained from Genzyme, Inc. (Cambridge, MA). The working solution of IL-4 that gave the maximal response (10–50 ng/ml; three different lots) contained less than 10 pg/ml of endotoxin as determined in a semi-quantitative E-tosyl assay kit (Sigma Chem. Co.).

**Monoclonal Antibodies**

The anti-E-selectin (H18/7, IgG2a) (5), anti-ICAM-1 (Hu5/5, IgG1) (35), and anti-HLA-A,B (W6/32, IgG2a) (35, 36) mAb were used as purified Fab' fragments at saturating concentration (25 μg/ml) in adhesion blocking studies and as hybridoma culture supernatant fluid for surface immunofluorescence assays. Murine mAb directed against distinct epitopes of L-selectin (anti-LAM1, all of the IgG1 isotype) were used as purified IgG at 10 μg/ml (54–56). The anti-VCAM-1 mAb IB/6 (IgG1) (41) and Hu 8/4 (58) were used as purified IgG (20 μg/ml) and Fab' (20 μg/ml), respectively. mAb TS1/18.1 (IgG1) (Amer. Type Culture Collection, Rockville, MD, clone HB 205) recognizes the β2-integrins (CD11/CD18) and was used as purified IgG (50 μg/ml) (47). Murine mAb directed to CD14 (63D3) was obtained from ATCC (clone 44-HB) and used as diluted ascites fluid (1:300) or purified IgG in indirect immunofluorescence assays. Murine mAb 1H18 (IgG1; 1:300 dilution of ascites) recognizes αβ1 and was the gift of Dr. Brad McHenry, Baylor College of Medicine, Houston, TX (49).

Rat monoclonal antibody MAB13 (IgG3) (1) recognizes human β1-integrins (CD29) and was used as purified IgG (25 μg/ml). Rat mAb M182/8.8 (IgG3) recognizes murine but not human CD18 (ATCC, clone TIB-218) and was used as a control isotype matched non-binding mAb (purified IgG) in adhesion assays.

**Cell Culture**

HUVEC were isolated from two to five umbilical cord veins, pooled, and established as primary cultures in M199 containing 20% FCS (Hyclone Labs., Urem., UT) (34, 35). Primary HUVEC cultures were serially passaged (1:3 split ratio) and maintained in M199 containing 10% FCS, endothelial cell growth factor (50 μg/ml), Biomedical Technologies, Inc., Stoughton, MA), porcine intestinal heparin (50 μg/ml, Sigma Chem. Co., St. Louis, MO), and antibiotics. For experimental use, HUVEC (passage 1-2) were plated on 25 × 75 mm permanox plastic tissue culture slides (American Bioanalytical, Natick, MA) or glass microscope slides (Fisher Scientific, Medford, MA) coated with human fibronectin (2 μg/cm²; Collaborative Research, Bedford, MA) and grown within a 2-cm diamircle delineated by a ring of 12 M polystyrene. For adhesion studies in the flow plate apparatus, 25-mm glass coverslips (No. 1 thickness, Thomas Scientific, Swedesboro, NJ) were immersed in 0.1 NaOH for 1 h and extensively rinsed in distilled H2O. The coverslips were dipped in absolute ethanol, flamed in a bunsen burner to evaporate the ethanol and coated with 2 μg/cm² human fibronectin. HUVEC (subculture 1-2) were plated at constant density and incubated for 24 h before cytotoxic lysis lysis. For surface immunofluorescence studies, HUVEC were plated on 0.1% gelatin-coated or fibronectin-coated plastic wells of 96 well microtiter plates (Costar Corp., Cambridge, MA). Experiments were initiated once HUVEC were confluent (~24 h).

**Leukocyte Isolation and Culture**

Human monocytes were isolated from anticoagulated whole blood or from plateletpheresis residues by centrifugation on Ficoll-Hypaque density gradient centrifugation at 15 °C (LSM, Organan Teknika, Durham, NC) followed by counterflow centrifugation elutriation on a Beckman J2-21 M/E centrifuge using a JE-6 elutriation rotor and a 6-ml Sandernex chamber (Beckman Instruments, Palo Alto, CA). The method developed by Doherty et al. (14, 15) was used with the following modifications. The elutriation buffer was DPBS supplemented with 3 mM EDTA and 0.25% HSA. The elutriation rotor and harness was assembled, sterilized with chloroz bleach, and flushed with 1,000 ml of sterile DPBS. The mononuclear cell fraction isolated by Ficoll-Hypaque density gradient (3 × 10⁶ cells) was loaded at 14 ml/min onto the elutriator centrifuge rotor head (2,500 ± 10 rpm at 10°C) and fractions of elutriated cells were collected at increasing flow rates (14, 15). Monocytes eluted at 21.5 ml/min. Monocyte suspensions were 89 ± 4% (N = 17) pure with 8% lymphocyte, <2% granulocyte, and 2% monocytes/macrophages. The washed monocytes were resuspended in cold DPBS containing 0.75 mM Ca²⁺ and 0.75 mM Mg²⁺ and 0.2% HSA. Monocytes were resuspended in cold DPBS containing 0.75 mM Ca²⁺ and 0.75 mM Mg²⁺ and 0.2% HSA. Monocytes were eluted at 21.5 ml/min and flushed with 1,000 ml of sterile DPBS. The mononuclear cell fraction isolated by Ficoll-Hypaque density gradient (3 × 10⁶ cells) was loaded at 14 ml/min onto the elutriator centrifuge rotor head (2,500 ± 10 rpm at 10°C) and fractions of elutriated cells were collected at increasing flow rates (14, 15). Monocytes eluted at 21.5 ml/min. Monocyte suspensions were 89 ± 4% (N = 17) pure with 8% lymphocyte, <2% granulocyte, and 2% monocytes/macrophages.
U937 cells were obtained from the ATCC and were maintained in RPMI-1640 with 25 mM Hepes containing 10% FCS, 1 mM L-glutamine and antibiotics. U937 cells stably expressing human L-selectin were prepared by electroporation of cells with 40 μg of the pLAM-1 cDNA (59) in the pZIP-neo SV40 vector. Transfectants were selected in medium containing 0.5 mg/ml G418. Murine L-cells were obtained from ATCC and were maintained in DMEM with 10% FCS. L-cells stably expressing the 7-domain form of human VCAM-1 were prepared by electroporation of cells with 20 μg of the pLAM-1 cDNA in a modified SV40 vector. Transfectants were selected in DMEM-10% FCS containing 1 mM L-glutamine and incubated at 37°C with assay media alone or media containing saturating concentrations of various HUVEC-directed or leucocyte-directed mAb for 30 min. In parallel, leukocytes (4 × 10^7) were centrifuged (300 g, 5 min at 4°C) and suspended in cold assay buffer (100 μl, total volume) containing leucocyte-directed or HUVEC-directed mAb or both for 20 min. Leukocyte suspensions were added to HUVEC monolayers that were rotating at 64 rpm at 37°C in a humidified condition at 37°C. After incubation for 8 min, the medium was gently removed and the slides were placed vertically in ice-cold fixative (1% glutaraldehyde (Sigma) in PBS, pH 7.4) and stored overnight at 4°C. The number of adherent monocytes was determined by counting 6 different fields using an ocular grid and 20× objective (0.16 mm^2 per field). Fields for counting adherent leukocytes were located at half radius distances from the center of the monolayers. The percent inhibition was calculated as previously described (55).

Endothelial-Leukocyte Attachment Assay

Attachment assays were carried out under rotation (64 rpm) as previously described in detail (55, 56). Briefly, confluent HUVEC were stimulated for 24 h (or as otherwise stated) with culture media containing an optimal concentration of rhIL-4 (Genzyme, Inc., Boston, MA). Before assay, HUVEC monolayers were washed three times with assay media (RPMI-1640 containing 5% FCS and 1 mM L-glutamine) and incubated at 37°C with assay media alone or media containing saturating concentrations of various HUVEC-directed or leucocyte-directed mAb for 30 min. In parallel, leukocytes (4 × 10^7) were centrifuged (300 g, 5 min at 4°C) and suspended in cold assay buffer (100 μl, total volume) containing leucocyte-directed or HUVEC-directed mAb or both for 20 min. Leukocyte suspensions were added to HUVEC monolayers that were rotating at 64 rpm at 37°C in a humidified condition at 37°C. After incubation for 8 min, the medium was gently removed and the slides were placed vertically in ice-cold fixative (1% glutaraldehyde (Sigma) in PBS, pH 7.4) and stored overnight at 4°C. The number of adherent monocytes was determined by counting 6 different fields using an ocular grid and 20× objective (0.16 mm^2 per field). Fields for counting adherent leukocytes were located at half radius distances from the center of the monolayers. The percent inhibition was calculated as previously described (55).

Endothelial-Leukocyte Interactions in a Parallel Plate Flow Chamber

Apparatus Design. The parallel-plate flow chamber used in this study has been described in detail (50). Briefly, the chamber is composed of two stainless steel plates separated by a silastic gasket (250 μm thickness, Dow Corning). The flow channel is formed by removal of a 5.0 × 50.0 mm rectangular section from the silastic gasket. Defined levels of flow are applied to the endothelial monolayer by perfusing media containing 0.75 mM Ca^2+ and Mg^2+ and 0.2% HSA through the channel using a syringe pump (Harvard Apparatus, Natick, MA). A copper heating plate with two electrical heating cartridges (SC12-1, Hotwatt, Danvers, MA) was mounted on the top of the chamber to maintain temperature at 37°C. The channel flow can be approximated as two-dimensional fully developed laminar flow with a simple parabolic velocity profile since the channel height is a linear function of the volume flow rate through the channel. Various wall shear stress levels were achieved with the following flow rates: 0.4 dynes/cm^2, 0.2 ml/min; 0.8 dynes/cm^2, 0.4 ml/min; 1.8 dynes/cm^2, 0.85 ml/min; 4.4 dynes/cm^2, 2.2 ml/min.

Experimental Application. Confluent endothelial monolayers on glass coverslips were incubated with culture media containing rhIL-4 or culture media alone for 24 h. Before assay, HUVEC monolayers were incubated with culture media containing various mAb or culture media alone for 30 min at 37°C, and then carefully positioned in a circular recess in the bottom plate of the chamber where a portion (50 × 250 mm) of the monolayer was exposed to flow. Care was taken to eliminate air bubbles in the channel during loading of the coverslip and assembly of the flow chamber as previously detailed (50). The flow apparatus with a HUVEC monolayer mounted on an inverted microscope (Nikon Diaphot-TMD, Melville, NY) equipped with 10× and 40× phase contrast objectives. A circular glass window in the top plate allows direct live time microscopic examination of the monolayer exposed to flow. The monolayer was perfused for 5–10 min with perfusion media to verify that the monolayer was confluent and intact. Currently, EHUCV monolayers or U937 cells (3 × 10^7) were incubated with various mAb for 10 min at 4°C and diluted with perfusion media to 10^4 cells/ml. The mAb concentration was adjusted to saturating levels in the perfusion buffer and leukocytes were perfused through the chamber at controlled flow rates. The entire period of perfusion was recorded on videotape using a video camera and a video recorder (Panasonic model AG-6750A) equipped with a time-date generator with a millisecond clock (Panasonic model WJ-810). Leukocyte adhesion was determined after 10 min of perfusion by analysis of 12–15 high power (40×) fields from videotape. Leukocytes were considered to be adherent after 30 s of stable contact with the monolayer and usually were well spread on the luminal surface of the endothelium. Since ~8% of the cell suspension was lymphocytes, we determined whether lymphocytes attached to IL-4-activated HUVEC under flow at 18 dynes/cm^2. Lymphocyte adhesion was very low (3 ± 2 lymphocytes (98 ± 1% pure) per 40× field (~75 cells/mm^2)) whereas monocyte adhesion was quite large (35–50 monocytes/field (750–1,000/mm^2)). Thus, based on these data and the fact that contaminating lymphocytes make up 8% of the monocyte suspension used in our studies, the overall contribution of lymphocytes would be small. For calculation of monocyte-endothelial interactions, rolling monocytes were not considered as "adherent". The velocities of 50–70 consecutive leukocytes in the field of focus flowing past a 2,500 μm^2 area were determined at multiple time points during perfusion by measurement of time required to travel 20 μm in multiple frames of videotape. The 2,500 μm^2 area for view on the video screen is formed by a solid mask placed over the entire video screen with an area removed from the center to expose a portion of the microscopic field (50 × 50 μm). Distances were calibrated for each experiment using a Neubauer cell counting chamber grid or stage micrometer. Leukocytes rolling on the endothelium were easily visualized since they travel more slowly (~80 μm/s) than free-flooding cells.

Immunofluorescence Assays. Indirect immunofluorescence assays for L-selectin, ICAM-1, CD11b/CD18 and CD11a surface expression on leukocytes were performed as previously reported (54) using mAb LAM-1-3, TS1/18 and TS2/13, respectively, that were a two-step detection protocol using FITC-conjugated goat anti-mouse F(ab’)2 (Caltag, S. San Francisco, CA). The fluorescence of 10,000 cells was measured on a FACScan (Becton Dickinson) and the data are presented as mean channel fluorescence (4 decade log scale) vs cell number. Statistics. The adhesion data was collected using ANOVA and Student's two sample t-test was used to calculate statistical significance. ANOVA and Mann-Whitney-Wilcoxon rank sum test and confidence interval analysis were performed on velocities of monocytes on control or IL-4-activated HUVEC (Minitab statistical software, release 7, Minitab Inc., State College, PA). p values < 0.05 were considered significant.

Results

Elutriated Monocytes Maintain Expression of L-Selectin and CD11b

To obtain monocytes without loss of L-selectin, we used the elutriation strategy devised by Doherty and coworkers (14, 15) that requires only a short (~20 min) elutriation protocol to obtain purified monocytes. Isolation of monocytes from peripheral blood mononuclear leukocytes by this protocol resulted in minimal alterations in cell surface L-selectin or CD11b expression as documented by indirect immunofluorescence analysis (Fig. 1). Such monocytes were used in all experiments reported.

IL-4 Treatment of HUVEC Monolayers Induces VCAM-1 Surface Expression but Does Not Alter ICAM-1 or Induce E-Selectin Surface Expression

Previous reports have shown that rhIL-4 selectively activated HUVEC to express VCAM-1 without altering ICAM-1 expression or inducing E-selectin expression (49, 60). We confirmed these findings in our model system using surface immunofluorescence assays and found that VCAM-1 expression was maximal after 24 h of treatment (data not shown). Based on these observations, subsequent experiments were performed using 24 h treatments with IL-4.
CD18 (mAb TS1/18)  L-selectin (mAb LAM1-3)

**Figure 1.** Monocyte expression of L-selectin and CD18. Peripheral blood mononuclear leukocytes isolated on density gradients (Top, a and c) and elutriated monocytes (Bottom, b and d) were incubated with mAb TS1/18 or LAM1-3 to assess the surface expression of CD11/CD18 and L-selectin, respectively, using indirect immunofluorescence flow cytometric analysis (solid lines). The FITC fluorescence intensity (four decade scale) of monocytes stained with an isotype-matched non-binding control (mAb K16/16 (4) IgG1) is indicated by a broken line. Data presented were essentially identical to that obtained in seven separate preparations.

**IL-4-activated Endothelium Supports Increased Peripheral Blood Monocyte Adhesion under Rotating Conditions**

Adhesion of peripheral blood monocyte to unactivated HUVEC monolayers was low under rotating conditions (75 ± 46 monocytes/field, mean ± SD, N = 13). In contrast, IL-4 treatment of human HUVEC monolayers for 24 h promoted a sevenfold increase in monocyte adhesion to 527 ± 111 monocytes/field (N = 12). Adhesion studies with HUVEC monolayers incubated for 24 h with varying concentrations of IL-4 revealed a dose-dependent increase in monocyte adhesion that was significantly increased at 0.1 ng IL-4/ml and reached a plateau at 10 ng IL-4/ml (data not shown).

**An IL-4 Inducible Endothelial Ligand for L-Selectin in Conjunction with VCAM-1 Mediate Monocyte Adhesion under Rotation**

Increased adhesion of monocytes to IL-4-activated endothelium was detectable by 4 h, increased at 24 h, and was sustained for at least 48 h (Fig. 2). When adhesion assays were performed with endothelium activated with IL-4 for various times in the presence of function blocking anti-L-selectin mAb (LAMI-3), adhesion was significantly reduced at each time point. In parallel, anti-VCAM-1 mAb (E1/6) also significantly inhibited adhesion, and the combination of anti-L-selectin and anti-VCAM-1 blocked essentially all cytokine-induced monocyte adhesion at each time point. These data indicate that an activation-dependent L-selectin ligand(s) is induced on the endothelial cell surface by 4 h and expression is sustained for up to 48 h.

**Using a panel of mAb that recognize spatially and functionally distinct epitopes on L-selectin (54), blocking studies were carried out to determine the epitopes on L-selectin involved in monocyte attachment to IL-4-activated HUVEC. Anti-LAMI-1, -3, -4, -6, and -7 mAb, which block lymphocyte (56) binding to high endothelial venules (HEV), and lymphocyte and monocyte adhesion to TNF-activated endothelium (55), also inhibited monocyte attachment to IL-4-activated endothelium (46, 62, 75, 55, and 55% inhibition, respectively). mAb that do not block lymphocyte and monocyte attachment to HEV or HUVEC (LAMI-5, -10, -11) also failed to block monocyte attachment to IL-4-activated HUVEC. Thus, similar epitopes of L-selectin are involved in monocyte and lymphocyte adhesion to endothelium.

**IL-4-activated Endothelium Supports Monocyte Adhesion and Spreading under Defined Laminar Flow**

Monocyte-endothelial interactions were examined under defined flow in an in vitro model system (50). Monocyte adhesion to unactivated HUVEC monolayers declined as the wall shear stress increased, and few monocytes attached above 0.8 dynes/cm² (Fig. 3). In contrast, monocytes bound well to IL-4-activated endothelial monolayers at levels of wall shear stress up to 1.8 dynes/cm²; however no adhesion occurred at 4.4 dynes/cm². Based on these data, all subsequent studies were carried out at 1.8 dynes/cm².

Examination of monocyte-endothelial interactions revealed that a significant number of monocytes rolled on IL-4-acti-
Figure 3. Monocyte adhesion to endothelial monolayers under defined flow conditions. Confluent IL-4-activated or -unactivated HUVEC monolayers were prepared and inserted into the flow chamber, and monocytes were perfused through the chamber at various flow rates (resulting in the estimated wall shear stresses indicated) as detailed in Materials and Methods. Data are from three separate experiments.

Table 1. Monocyte Rolling Velocities on Interleukin-4-activated Endothelium under Flow

| HUVEC treatments/mAb | Monocyte rolling velocities (µm/s) |
|----------------------|-----------------------------------|
| Unactivated, media   | 205 ± 53                          |
| IL-4-activated, media| 78 ± 18*                          |
| W6/32 (HLA-A,B complex) | 83 ± 14†                         |
| LAM1-3 (L-selectin)   | 303 ± 69‡                         |
| LAM1-14 (L-selectin)  | 79 ± 40†                          |
| MAB 13 (CD29, β1-integrins) | 109 ± 30‡                       |
| 19H8 (αβ2)            | 93 ± 19†                          |
| E1/6 (VCAM-1)         | 87 ± 20‡                          |
| TS 1/18 (CD18, β2-integrins) | 77 ± 18†                        |

Monocyte rolling velocities were determined as detailed in Materials and Methods.
* Monocyte velocities were significantly lower on IL-4-treated HUVEC vs control HUVEC (p < 0.001).
† mAb W6/32, LAM1-14, MAB13, E1/6, and TS1/18 did not significantly alter monocyte rolling velocities as compared to media-treated IL-4-activated HUVEC.
‡ LAM1-3 significantly increased monocyte rolling velocities (p < 0.001) as compared to treatments with media or non-function blocking LAM1-14 mAb.

Figure 4. Monocytes roll, arrest, spread, and transmigrate across IL-4-activated endothelial monolayers under laminar flow. Monocytes were perfused across 24 h IL-4-activated HUVEC monolayers at 1.8 dynes/cm². Sequential video frames depicting monocyte rolling (arrowhead and arrow, a–c), arrest (d, arrowhead), spreading (e, arrowhead), and transendothelial migration (f, arrowhead) were digitized. The appropriate areas of interest from each digitized video frame were combined using commercial software to create the composite image. This composite image shows the various stages that occur between monocytes and activated endothelial monolayers under defined flow at 1.8 dynes/cm². The images a–f were taken from video tape 2, 2, 2, 18, and 33 s apart (∼1 min elapsed time).

L-Selectin Facilitates Monocyte Adhesion Via αβ2-VCAM-1 under Flow

The contributions of both L-selectin- and VCAM-1-dependent adhesion pathways to monocyte attachment and rolling on IL-4-activated HUVEC were evaluated at a wall shear stress of 1.8 dynes/cm². In agreement with the data ob-
monocytetype adhesion under flow.

The role of both L-selectin and VCAM-1-αβ4 interaction in monocyte adhesion and arrest under flow could be easily distinguished both visually and by measuring rolling velocities of monocytes incubated with appropriate mAb. mAb directed to αβ4, or VCAM-1 did not significantly alter the velocities of monocytes rolling on IL-4-activated HUVEC (Table I), but did block arrest (Table II). Anti-L-selectin mAb (LAM1-3) significantly inhibited monocyte rolling and thereby, inhibited >90% of attachment. In the presence of mAb to either L-selectin or VCAM-1-αβ4, few if any slow rolling (1-10 μm/s) monocytes were observed. Taken together, the data suggest that L-selectin interacting with its inducible endothelial ligand allows monocytes to de-
celerate to rolling velocities that facilitate the interaction of αβ4 with VCAM-1, which in turn, mediates arrest on the activated endothelial surface.

The ability of αβ4-VCAM-1 pathway alone to mediate stable attachment of monocytes under flow was examined using monolayers of L-cells stably expressing the seven domain form of VCAM-1. By quantitative fluorescence immunobinding assay, these L-cell transfectants uniformly express VCAM-1 at levels comparable to that expressed by IL-4-activated endothelium and supported VCAM-1-dependent adhesion (see Materials and Methods). At 1.8 dynes/cm², monocytes did not roll on or attach to L-cell-VCAM-1 monolayers. Moreover, no adhesion or rolling was observed at 0.8 dynes/cm² with either L-cell-VCAM-1 or untransfected L-cell monolayers. Thus, αβ4 interactions with VCAM-1 stably expressed on murine L-cells was not sufficient to support monocyte adhesion under flow in this system.

### Table II. Monoclonal Antibody Inhibition of Monocyte Adhesion under Flow

| HUVEC treatments/mAb       | (N) Adherent monocytes/mm² | Inhibition of adhesion (%) |
|----------------------------|----------------------------|---------------------------|
| Unactivated, media        | 7, 53 ± 82                 | -                         |
| IL-4 activated, media     | 3, 677 ± 169               | -                         |
| W6/32 (HLA-A,B complex)   | 6, 821 ± 241               | -                         |
| LAM1-3 (L-selectin)        | 4, 134 ± 46*               | 89*                       |
| LAM1-14 (L-selectin)       | 2, 1273 ± 156              | 0                         |
| E1/6 (VCAM-1)             | 3, 77 ± 109*               | 97*                       |
| Hu 8/4 (VCAM-1)           | 1, 961 ± 224               | 0                         |
| MAB 13 (CD29, β1-integrins)| 2, 26 ± 6*                 | 100*                      |
| 19H8 (αβ4)                | 2, 141 ± 91*               | 89*                       |
| TS 1/18 (CD18, β integrins)| 3, 909 ± 146              | 0                         |

Confluent control or IL-4 activated HUVEC monolayers were washed four times, incubated with various mAb for 30 min at 37°C, and then inserted into the flow chamber. Concomitantly, monocytes (12 × 10⁶ in 1 ml perfusion media) were incubated with media alone or media containing various control or function blocking mAb for 20 min, diluted to 12 ml, and then perfused across the HUVEC monolayer. All studies were carried out at a wall shear stress of 1.8 dynes/cm². N = number of separate experiments. Firmly adherent cells were determined (40× objective) at the end of 10 min of monocyte perfusion (rolling cells were not included).

* Monocyte adhesion was significantly inhibited by mAb LAM1-3 (p < 0.0011), E1/6 (p < 0.0007), MAB13 (p < 0.0005), and αβ4 (p < 0.015) as compared to control mAb W6/32 (anti-HLA-A,B).

Figure 5. U937-LAMI cell adhesion to endothelial monolayers under defined flow conditions. Confluent IL-4-activated or unactivated HUVEC monolayers were prepared and inserted into the flow chamber, and stably transfected U937-LAMI cells were perfused through the chamber at various flow rates as detailed in Materials and Methods. Data are from two separate experiments.
was similar to that of blood monocytes (Fig. 5). In contrast, U937 cells did not attach to control or IL-4-activated HUVEC at either 1.8 or 0.8 dynes/cm² (N = 3 experiments). These data imply that the combination of L-selectin and #α#β# interacting with their respective endothelial ligands is necessary and sufficient for rolling and arrest under flow conditions.

Further experiments examined U937-LAM1 rolling and attachment to IL-4-activated endothelium in the presence of appropriate mAb. In three separate experiments, the effects of function blocking mAb on U937-LAM1 cells were essentially the same as that for monocytes. Anti-L-selectin mAb caused a significant increase in rolling velocities (LAM1-3, 332 ± 55 μm/s, mean ± SD, vs media control, 99 ± 34 μm/s; p < 0.002) and totally inhibited upregulated U937-LAM1 adhesion as compared to media control (Table III). Similarly, anti-CD29 and anti-VCAM-1 mAb blocked all upregulated U937-LAM1 adhesion but had no significant effects on U937-LAM1 rolling (velocities with anti-CD29, 112 ± 14 μm/s). These results corroborate the data obtained with monocytes, and demonstrate that stable surface expression of L-selectin in U937 cells (αβ and L-selectin positive) confers the ability to roll on, and stably bind to, IL-4-activated endothelium under flow.

β# Integrins Mediate Monocyte Spreading but Not Rolling or Attachment under Flow

Treatment of monocytes with function blocking anti-CD18 mAb (TS1/18) consistently had no effect on monocyte rolling velocities or attachment to IL-4-activated HUVEC (Tables I and II). However, anti-CD18 mAb did reduce the number of adherent monocytes which spread or transmigrated across the endothelial cell monolayer. The video images in Fig. 6 show the extent of monocyte adhesion and spreading to IL-4-activated HUVEC in the presence of LAM1-14 (non-function blocking L-selectin), anti-β# integrin (TS1/18) or anti-αβ, (19H8) mAb (Fig. 6, a–c, respectively). Many monocytes attached and spread (depicted by arrows) to activated endothelium treated with control LAM1-14 mAb (Fig. 6 a; 26 ± 5% of adherent monocytes were spread/transmigrated, N = 124 monocytes counted in seven separate fields with

| Table III. Monoclonal Antibody Inhibition of U937-LAMI Adhesion under Flow |
|-----------------|---------------------------------|-------------------------------|
| HUVEC treatments/mAb | Adherent U937-LAMI/mm² | Inhibition of adhesion (%) |
| Unactivated, media | 63 ± 42 | 630 ± 99 |
| IL-4-activated, media | 21 ± 21 | 21 ± 21 |
| LAM1-3 (L-selectin) | 63 ± 44 | 100* |
| El/6 (VCAM-1) | 10 ± 15 | 100* |
| MAB13 (CD29) | 10 ± 15 | 100* |

Confident control or IL-4-activated HUVEC monolayers were prepared as detailed in Table II. U937-LAMI (12 × 10⁶ in 1 ml perfusion media) were incubated with media alone or media containing various mAb, diluted to 12 ml, and then perfused across the HUVEC monolayer. All studies were carried out at wall shear stress of 1.8 dynes/cm². The data are representative of two separate experiments. Adherent cells were determined as in Table II. * U937-LAMI cell adhesion was significantly inhibited (p < 0.03) by mAb LAM1-3, El/6, and MAB13 as compared to media-treated IL-4-activated HUVEC.

Figure 6. Anti-β# integrins inhibit spreading of attached monocytes but not rolling or attachment under flow. Various frames of video tape recordings of monocyte adhesion to endothelium under flow were digitized and photographed. Monocytes were perfused across IL-4-activated endothelial monolayers in the presence of control non-blocking anti-L-selectin mAb (LAM1-14) (a), function blocking mAb directed to β# integrins (TS1/18) (b), or function blocking anti-αβ, (19H8) (c) and the experiments recorded on videocassette using a Nikon TMD inverted microscope and a 40× phase contrast objective. The tapes were replayed and selected portions of the video frames were digitized and photographed. Arrows indicate spread/transmigrated monocytes.
Discussion

To our knowledge this is the first report to examine the cellular processes and molecular mechanisms of monocyte-endothelial adhesive interactions under defined flow conditions in vitro. A recent model of leukocyte-endothelial interactions has proposed that this process requires multiple, sequential steps (7). The initial contact between the leukocyte and endothelial cell surface at normal levels of blood flow is mediated primarily by selectins interacting with their carbohydrate ligands. This interaction appears to be reversible unless the second phase, leukocyte activation, is initiated. Activation may occur by appropriate chemoattractants or accessory molecules that trigger surface adhesion molecules to become "activated" (i.e., change their avidity for counter-receptors/ligands [53]). Once activated, these molecules can engage their endothelial counter-receptors, and thus, initiate phase three, firm adhesion (arrest). Using an in vitro flow chamber and function blocking mAb, we have extended this model in the present study and have identified, at the cellular level, the events necessary for monocyte arrest and transmigration across endothelium which has been selectively activated with IL-4 (and lacks E-selectin) (see Fig. 7). The data indicate that monocytes decelerate and roll on the activated endothelium via L-selectin interacting with its inducible ligand(s) (Phase I). This step is reversible. The next step involves monocyte αβ1 interacting with VCAM-1 (Phase II). It is not known if αβ1-VCAM-1 interaction requires cell activation, although previous studies have reported that VCAM-1-αβ1 can mediate adhesion at 4°C. These data imply that activation of monocyte αβ1 may not be necessary (53, 55, 56) in this in vitro system. Once the αβ1-VCAM-1 pathway is engaged, monocytes attach stably under flow conditions (Figs. 6 and 7). Adherent monocytes then spread on the apical surface of the endothelium in a process that involves β-integrins (Phase III). This step most likely requires activation (57) and presumably engages engagement of ICAM-1 and/or ICAM-2 on the endothelial surface. Once spread, monocytes migrate to intercellular junctions and diapedes between endothelial cells (Phase IV).

Recent studies suggest this latter process is dependent, in part, on homotypic interactions of PECAM-1 (CD31) expressed on both leukocytes and endothelium (39). Taken together, these observations suggest a more complex, sequential model for monocyte-endothelial interactions than previously appreciated.

The evidence supporting this model of monocyte-endothelial interactions under flow is as follows. First, consistent with previous studies (36, 49, 61), IL-4 treatment of HUVEC monolayers induced VCAM-1 expression, but did not alter ICAM-1 expression and did not induce E-selectin expression. Second, few monocytes adhered (<50 monocytes/mm²) to unactivated endothelium at wall shear stress levels > 0.8 dynes/cm². In contrast, IL-4 treatment of HUVEC monolayers induced a dramatic increase in monocyte adhesion at levels of wall shear stress up to 1.8 dynes/cm². The velocities of monocytes were reduced on the activated monolayers as compared to control HUVEC, and a significant fraction of these monocytes were rolling. Third, functional blocking mAb to either L-selectin, VCAM-1, or αβ blocked monocyte adhesion to activated endothelium under both non-static (rotation assay, 75% inhibition) and defined flow (90% inhibition at 1.8 dynes/cm²) conditions. Blockade of L-selectin function by mAb LAM1-3 caused an increase in monocyte rolling velocities to levels that were not statistically different from that of monocytes flowing across unactivated endothelium. Few, if any, rolling monocytes were observed with LAM1-3 treatment, whereas control non-blocking LAM1-14 had no inhibitory effect on monocyte rolling or adhesion (Tables I and II). These data are consistent with previous reports using neutrophils that demonstrated that L-selectin is crucial for rolling in vivo (32, 62) and accumulation at sites of inflammation (26, 64). In contrast, function blocking mAb directed to β-integrins or VCAM-1 did not alter rolling velocities but instead increased the number of rolling cells. Fourth, αβ appeared to be the principal β-integrin involved in stable adhesion under flow since an αβ-specific mAb (19H8) was nearly as effective as β-specific mAb (MAB 13) in blocking adhesion. Since αβ-specific mAb blocked >90% of adhesion, it is unlikely that other α-subunits of β-integrins, such as α5 or α6, are involved. Fifth, as was demonstrated in experiments with the monocyte-like U937 and U937-LAM1 cell lines, both adhesion pathways are necessary for rolling and adhesion. U937 cells, which express αβ, but not L-selectin (VLA4, L-sel⁻), do not roll on or attach to (<10 cells/mm²) IL-4-activated endothelial monolayers at 1.8 dynes/cm² (Fig. 5). However, U937-LAM1 cells, which express both L-selectin and VLA4 (VLA4, L-sel⁺), rolled on and stably adhered to IL-4-activated endothelium at 1.8 dynes/cm². The characteristics of U937-LAM1 cell adhesive interactions with the activated
H.UVEC under flow were therefore similar to that of monocytes. Function blocking mAb specific for either $\alpha_\beta^1$, VCAM-1 or L-selectin, but not $\beta_2$-integrins (CD11b/CD18), blocked $>95\%$ of adhesion. Finally, monocytes did not roll on or attach to murine L-cell monolayers stably expressing VCAM-1 at levels comparable to IL-4-activated HUVEC, indicating that VCAM-1-$\alpha_\beta_1$ interactions alone are not sufficient to arrest monocytes at 1.8 dynes/cm$^2$.

This report provides the first clear indication that $\beta_1$- and $\beta_2$-integrins have distinct functions in monocyte adhesive interactions with the vascular endothelium. Specifically, $\beta_1$-integrins functioned in stabilizing the initial attachment under flow (arrest), whereas $\beta_2$-integrins were required for cell spreading and motility once the monocyte had stably bound to the apical surface of an endothelial cell. This is in contrast to neutrophils, which do not express $\alpha_\beta_1$-integrins and which use $\beta_2$-integrins to mediate firm attachment to endothelium (35, 51, 62). It is of interest that monocyte $\beta_1$-integrins are not used for arrest to IL-4-activated HUVEC, because $\beta_2$-integrins can clearly mediate this function for neutrophils. It is unlikely that IL-4-activated HUVEC do not transmit appropriate signals for activation of $\beta_1$-integrins, since $\beta_2$-integrins are involved in spreading. Neutrophils may use distinct $\beta_2$-integrins for arrest and spreading, thereby making up for the lack of $\beta_1$-integrins. Future studies examining monocyte interactions with TNF-$\alpha$ or IL-1$\beta$-activated HUVEC monolayers, which express elevated levels of E-selectin as well as ICAM-1 and VCAM-1, may provide additional insight into the molecular mechanisms of the differential function of $\beta_1$- and $\beta_2$-integrins in monocyte arrest under flow.

Recent reports have identified endothelial cell molecules from lymphoid tissues that may function as ligands for a chimeric L-selectin molecule. Lasky et al. (28) have cloned a cDNA from a murine lymph node cDNA library that encodes a serine/threonine-rich protein core, termed glyCAM-1 (glycosylation-dependent cell adhesion molecule), that functions as a ligand for L-selectin. Expression of this ligand was constitutive and restricted to peripheral lymph node and mesenteric HEV endothelial cells in mice; glyCAM-1 expression was not upregulated with an inflammatory stimulus. More recently this group has identified CD34, another mucin-like molecule, as a ligand for a L-selectin chimeric molecule (3). It will be important to determine the distribution and expression of this molecule in various tissues (both lymphoid and non-lymphoid and at peripheral vascular beds at sites of inflammation) and to determine its role in L-selectin dependent monocyte as well as other leukocyte cell type rolling on vascular endothelium. In addition, Salmi and Jalkanen (46) reported the immunopurification of a 90-kD ligand, termed VAP-1, from tonsillar extracts that supports lymphocyte adhesion. VAP-1 was absent from normal HUVEC and treatment with various cytokines (TNF-$\alpha$, IL-$1\beta$ and LPS) for 4 or 24 h did not induce expression of this molecule in cultured HUVEC. Thus, it appears that the ligand on an activated peripheral vascular endothelium for L-selectin is distinct from VCAM-1, E-selectin, and ICAM-1, and that it also is distinct from the constitutively expressed lymphocyte homing receptor ligand expressed on HEV endothelial cells glyCAM-1 or other previously described molecules (28). The results from our time-course study under rotation (Fig. 2) indicate that this endothelial ligand for L-selectin is induced by 4 h and remains elevated for at least 48 h. Previous studies have demonstrated that TNF-$\alpha$ or IL-1$\beta$ treatment of cultured HUVEC also induced an endothelial ligand(s) for L-selectin that contributes to lymphocyte, monocyte, and neutrophil adhesion (55, 56). At this time, we have no direct evidence that the L-selectin ligand(s) induced by IL-4, IL-1, or TNF-$\alpha$ are distinct, and the results from our mAb epitope mapping experiments and our previous studies (54, 55, 56) would suggest that similarities exist in the region of the L-selectin involved in interactions with the endothelial ligand for L-selectin. L-selectin interaction with its endothelial ligand(s) is an important adhesion pathway that facilitates monocyte attachment to activated endothelium; molecular characterization of vascular endothelial ligand(s) will be an important goal for dissecting the role of monocyte emigration in such inflammatory responses as transplant rejection, and in atherosclerosis.

The data presented in Fig. 1 indicate that elutriated monocytes are not significantly activated during the isolation protocol. This is an important consideration in the study of monocyte-endothelial interactions. Using a modification of the protocol of Doherty et al. (14, 15), monocytes were elutriated in less than 20 min and were consistently $>89\%$ pure with very low neutrophil or lymphocyte contamination and essentially no platelet contamination. Previous studies have demonstrated that during neutrophil and monocyte activation L-selectin is shed concomitant with a 2–5-fold increase in CD18 expression (18, 24, 26, 27, 51, 55, 57). Expression of L-selectin and CD18 on elutriated monocytes were essentially identical to monocytes in the mononuclear fraction isolated by density gradient. The preparations of monocytes used in these experiments also did not bind significant levels of mAb 15/7, which recognizes an activation epitope of $\alpha_\beta_1$ (Pizcueta, P., F. W. Luscinskas, and T. Yednoch, manuscript in preparation). Moreover, the basal adhesion of monocytes to unactivated endothelium under rotation or flow was consistently low, again suggesting that elutriated monocytes were not activated.

At 1.8 dynes/cm$^2$, most monocytes in contact with the activated endothelium were rolling at $\sim80 \mu m/s$, and some of these rolling monocytes had velocities in the range of 3–10 $\mu m/s$ (Fig. 4). This population of slowly rolling monocytes exhibited features similar to that previously described for neutrophils interacting with activated endothelium under flow (51): transient adhesion to endothelium ($<5$ s) followed by release and resumption of rolling at $\sim3–10 \mu m/s$. That monocytes roll rather than slide or tumble under flow was clearly distinguished under a 40x objective; the large nucleus rotated as the cell traveled across the surface of the endothelial monolayer. Another feature of monocyte-endothelial interactions under flow was that adherent monocytes locally altered the laminar flow profile. Initially, monocytes bound to endothelial cells in a random fashion, but once a monocyte bound, attachment of subsequent cells in the vicinity occurred preferentially just downstream from the first cell rather than to areas with no attached cells. This pattern of adhesion may be due to flowing monocytes following local disturbances in the flow above or behind adherent cells and/or because adherent cells protruded above the monolayer surface and therefore were in contact with a greater number of flowing monocytes. This alteration in laminar flow also has been observed with neutrophils interacting with immo-
bilized P-selectin (29). Finally, the pattern of monocyte attachment under increasing levels of wall shear stress (Fig. 3) indicates that between 1.8 and 4.4 dynes/cm², the proadhesive attachment forces generated by L-selectin interacting with its endothelial ligand(s) alone were unable to sustain monocyte contact with endothelium. Although the pump used with the flow plate apparatus was unable to produce flow rates intermediate between 1.8 and 4.4 dynes/cm², the data presented is sufficient to allow comparison to previous published results with neutrophils. Lawrence et al. (30) reported that neutrophil adhesion to unactivated endothelium was negligible above 1.0 dynes/cm² whereas neutrophil adhesion to IL-1- or LPS-activated endothelium was sustained at 2.0 dynes/cm², but was negligible at 3.0 dynes/cm². These results thus are similar to the data shown in Fig. 3 for monocytes.

Monocytes attached and spread on the luminal surface of the IL-4-activated endothelium under flow, but few migrated underneath the monolayer. In contrast, neutrophils readily transmigrated across TNF-α-activated HUVEC monolayer under flow (51, 52). In preliminary experiments using 6 h TNF-α-activated HUVEC, monocytes do roll, attach, and transmigrate in large numbers under the endothelial monolayer. This result suggests that the lack of emigration is not because monocytes are unable to migrate under flow in this system, but that IL-4 does not induce sufficient levels of endothelial accessory molecules or other factors which are necessary for transmigration. For example, Lo et al. (33) reported that E-selectin, which is induced by TNF-α, IL-1, and LPS but not IL-4, selectively activates neutrophil CD11b/CD18 function and migration, processes which could facilitate transmigration. IL-4 also induces approximately one-third the level of VCAM-1 expression and MCP-1 production under flow (51, 52). In preliminary experiments using 6 h TNF-α-activated HUVEC, monocytes do roll, attach, and transmigrate in large numbers under the endothelial monolayer. This result suggests that the lack of emigration is not because monocytes are unable to migrate under flow in this system, but that IL-4 does not induce sufficient levels of endothelial accessory molecules or other factors which are necessary for transmigration. For example, Lo et al. (33) reported that E-selectin, which is induced by TNF-α, IL-1, and LPS but not IL-4, selectively activates neutrophil CD11b/CD18 function and migration, processes which could facilitate transmigration. IL-4 also induces approximately one-third the level of VCAM-1 expression and MCP-1 production as compared to TNF-α (44) Ding, H., and F. W. Luscsinskis, unpublished observations.

In summary, using IL-4-activated HUVEC monolayers which selectively express VCAM-1 and an L-selectin ligand but not E-selectin, and appropriate function blocking mAb, we have examined monocyte-endothelial interactions in an in vitro flow model. We found that monocytes rolled on and stably bound to IL-4-activated endothelium under flow; L-selectin mediated monocyte rolling and facilitated α6β1-integrin-dependent arrest under flow. Subsequent monocyte spreading required β2 but not β1-integrins. These findings extend current models of leukocyte-endothelial recognition to reveal additional steps, mediated by specific adhesion receptors, involved in the interactions of blood monocytes with endothelium under defined flow conditions.

The authors gratefully acknowledge Mr. William Atkinson and Ms. Kay Case for providing cultured HUVEC and maintenance of the U937 cell line. We thank Dr. Kenneth M. Yamada, Laboratory of Developmental Biology; National Institutes of Health (NIH) for kindly providing monoclonal antibody MAB13 for our studies; Dr. Dennis Doherty (Denver, CO) for his technical advice on monocyte isolation by elutriation; and Dr. Robert Melder, Massachusetts General Hospital, Boston, MA, Dr. Mary Gerritsen (Miles, Inc., West Haven, CT), and Dr. David Milstone, Brigham and Women’s Hospital for assistance in generating the digitized images presented in this manuscript. We also thank members of the Vascular Research Division for helpful discussions during the course of this work.

This research was supported by grants HL-47646, PO1 HL-36028, CA-36167, CA-34184, CA-54464 and AI-26872 from NIH. T. F. Tedder is a Scholar of the Leukemia Society of America. P. Pizcueta is the recipient of a Fulbright Fellowship/Spanish Ministry of Education and Science (MEC) Fellowship.

Received for publication 29 September 1993 and in revised form 9 March 1994.

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