Reduced Expression of Junctional Adhesion Molecule and Platelet/Endothelial Cell Adhesion Molecule-1 (CD31) at Human Vascular Endothelial Junctions by Cytokines Tumor Necrosis Factor- α Plus Interferon- γ Does Not Reduce Leukocyte Transmigration Under Flow

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The combination of tumor necrosis factor (TNF)- α plus interferon (IFN)- γ has been shown previously to promote redistribution of platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD31), junctional adhesion molecule (JAM), and VE-cadherin away from lateral junctions of human umbilical vein endothelial cell monolayers. In parallel, neutrophil transmigration was significantly reduced. Because PECAM-1 and JAM have been implicated in leukocyte transmigration, the observed redistribution by cytokine activation was presumed to represent the mechanism causing decreased transmigration under static conditions. The current results confirm that culture of human umbilical vein endothelial cells with TNF- α plus IFN- γ caused a decrease in surface-expressed and junctional-localized JAM and PECAM-1, but did not cause decreased leukocyte transmigration in an *in vitro* flow assay. Furthermore, blocking monoclonal antibody to PECAM-1 still significantly reduced monocyte transmigration, demonstrating that it retains a functional role even though its levels were reduced and redistributed away from junctions, whereas a panel of monoclonal antibodies to JAM failed to reduce leukocyte transmigration. Given the alterations in junction protein location, permeability function was assessed. IFN- γ alone or TNF- α plus IFN- γ significantly increased permeability, but TNF- α alone did not, suggesting lack of correlation between transmigration and loss of permeability. In conclusion, cytokine ac-

tivation induced loss and redistribution of PECAM-1 and JAM away from lateral junctions, but per se does not negatively regulate either neutrophil or monocyte transmigration under flow. (*Am J Pathol* 2001, 159:2281–2291)

Cytokines are essential components of host defense during injury, inflammation, and immune responses. *In vivo* experiments have documented that an intradermal injection of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , or both cytokines induces expression of adhesion molecules in endothelium (E-selectin, ICAM-1) and a concomitant increase in leukocyte adhesion and recruitment into tissues.¹ Subsequently, *in vitro* studies have evaluated the effects of TNF- α and IFN- γ , alone or in combination, on cultured vascular endothelial function.^{2,3} TNF- α induces E-selectin, VCAM-1, ICAM-1, and L-selectin ligands and dramatically increases blood leukocyte adhesion and transmigration.⁴ IFN- γ increases ICAM-1 expression, but does not increase leukocyte adhesion or transmigration *in vitro* under static conditions.³ The combination of TNF- α and IFN- γ was similar to TNF- α with respect to the level of induction of adhesion molecule expression and leukocyte (neutrophil) adhesion. Paradoxically, one report found leukocyte (neutrophil) transmigration was reduced by 60 to 70% as assayed under static conditions.⁵ In addition, platelet/endothelial cell adhesion molecule-1 (PECAM-1), which is involved in transmigration⁶ in most but not all models,⁷ was dramatically

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redistributed from lateral junctions. More recently a newly described transmembrane immunoglobulin superfamily member, junctional adhesion molecule (JAM), which is enriched at lateral junctions and has been implicated in monocyte and neutrophil recruitment in some⁸⁻¹⁰ but not all¹¹ murine models of inflammation, was also found to be redistributed away from junctions by the combination of TNF- α and IFN- γ .¹² Based on the above finding, these authors suggested that change in cellular localization or in redistribution of both JAM and PECAM-1 after TNF- α and IFN- γ , plays an important role in regulating transmigration of leukocytes.^{5,12} However, there was no direct demonstration that the protein redistribution observed was responsible for diminished migration. Their results are in conflict with previous *in vivo* studies in which TNF- α and IFN- γ increased vessel permeability and caused leukocyte extravasation.¹

In this study, we used a well-characterized *in vitro* flow model of leukocyte-endothelial interaction to test the above premise that redistribution of PECAM-1 and JAM by TNF- α and IFN- γ negatively regulates human blood neutrophil and monocyte transmigration. The flow model establishes two important criteria: first, that adhesive interactions occur under flow conditions that simulate small venules *in vivo*, and second, allows live-time continuous visualization of cell-cell interactions.¹³ The current results demonstrate that 24-hour pretreatment with TNF- α and IFN- γ does not lead to a decrease in adhesion or transmigration of either neutrophils or monocytes under flow, even though surface PECAM-1 and JAM are significantly reduced and redistributed away from endothelial lateral junctions. We suggest that conditions of defined fluid shear may impart as yet unidentified information to either leukocytes or endothelium that is otherwise lacking under static conditions. We also observe that the permeability of endothelial cell monolayers increases (loss of barrier function) with either IFN- γ alone or combined TNF- α plus IFN- γ treatment but is minimally altered by TNF- α alone. Interestingly, although treatment with IFN- γ alone results in disruption of barrier function, there is no effect on the junctional localization or total expression of JAM, PECAM-1, or VE-cadherin. We conclude that for cytokine-activated endothelium, the physical localization and density of molecules implicated in transmigration and in barrier function may not accurately predict the level of leukocyte transmigration under flow conditions.

Materials and Methods

Materials

Human recombinant TNF- α was obtained from Genzyme (Cambridge, MA) and was free of detectable endotoxin as reported previously.¹³ A concentration of 25 ng/ml for 24 hours induced expression of VCAM-1 and ICAM-1 while E-selectin expression had declined to ~30 to 50% of peak expression at 4 hours. Recombinant IFN- γ (produced in *Escherichia coli*, specific activity of 2×10^7 U/mg; working concentration was 100 U/ml) was obtained from Genentech (S. San Francisco, CA) and was

essentially free of endotoxin at the concentrations used. Hanks' balanced salt solution (HBSS) with (HBSS⁺) or without Ca²⁺ and Mg²⁺ (HBSS⁻), M199, RPMI 1640, and DPBS with or without divalent cations were obtained from BioWhittaker (Walkersville, MD). Fetal bovine serum was obtained from Hyclone (Urem, UT). All other chemicals were of the highest grade available from Baker Chemical (Phillipsburg, NJ). All buffers that came in contact with neutrophils or monocytes were purchased commercially and any subsequent manipulations of cells were performed in sterile disposable plasticware to minimize endotoxin contamination.

Monoclonal Antibodies (mAbs)

All monoclonal antibodies were used as purified IgG (unless noted otherwise) at saturating concentration as assessed by indirect immunofluorescence and flow cytometry. Murine mAbs directed to human JAM (J3, J10, 2A9, all IgG₁) were prepared as recently described in detail¹⁴ and used at 10 μ g/ml. A F(ab)₂ preparation of J10 also was tested in blocking studies at 10 μ g/ml mAb F11 against human JAM¹⁵ (kindly provided by Dr. John Hartwig, Brigham and Women's Hospital, Boston, MA) was used as purified IgG at 10 μ g/ml. Murine anti-ICAM-1 mAb (Hu5/3, IgG₁, function blocking, 10 μ g/ml) and anti-VCAM-1 (E1/6, IgG₁, function blocking, 1:200 dilution of ascites; Hu8/4, nonblocking, 10 μ g/ml, IgG₁) have been reported previously.¹⁶ Function blocking mAbs TS1/18 (IgG₁) or HP2/1 (IgG₁) recognize human CD11/CD18 (common β 2-integrin) and VLA-4 (α 4-integrin), respectively, and were used at 10 μ g/ml.¹⁶ A blocking mAb to PECAM-1 (Hec-7) was the kind gift of Dr. William Muller (Weill Medical College, Cornell University, NY) and has been shown previously to block monocyte transmigration under static⁶ or flow conditions.¹⁷ Control mAb W6/32 (IgG_{2a}) recognizes human class I Ag, which is expressed at high levels on human umbilical vein endothelial cells (HUVECs) and leukocytes, and does not alter leukocyte adhesion to cytokine-activated endothelium.^{13,18} p96, recently identified as endoglin, is a transforming growth factor β -binding protein that is constitutively expressed at high levels by endothelium and is not affected by various cytokine treatments.¹⁹

Culture of HUVECs

HUVECs were isolated from two to five umbilical cord veins, pooled, and established as primary cultures in M199 containing 20% fetal bovine serum.¹³ Primary HUVEC cultures were passed serially (1:3 split ratio) and maintained in M199 containing 10% fetal bovine serum, endothelial cell growth factor (50 μ g/ml; Biomedical Technologies, Inc., Stoughton, MA), porcine intestinal heparin (100 μ g/ml; Sigma Chemical Co., St. Louis, MO), and antibiotics. For use in the flow apparatus, HUVECs (subculture 1) were plated at 80% confluence on 25-mm circular glass coverslips (no. 2 thickness; Carolina Biological Supply, Burlington, NC) previously precoated overnight with human fibronectin (1 μ g/cm²). HUVECs

were allowed to reach confluence and then were used in experiments within 24 to 48 hours.

Leukocyte Isolation

Human monocytes were isolated from plateletpheresis residues by centrifugation on density gradients (LSM; Organon Teknika, Durham, NC) followed by counterflow centrifugation elutriation.²⁰ Monocyte suspensions were >91% pure with 6 to 8% lymphocyte, <2% granulocytes, and essentially no platelet contamination as determined by light scatter (FACScan; Becton Dickinson, Mountain View, CA) and flow cytometric analysis of cell surface antigens as previously described.²⁰ Polymorphonuclear leukocytes (neutrophils) were isolated from whole blood obtained from healthy human volunteers by venous puncture in accordance with the regulations of the Human Use Committee, The Brigham and Women's Hospital as previously described.²¹ Neutrophils were 95% pure as determined by Wright-Giemsa stain.

Leukocyte Adhesion and Transmigration

Flow Assay

Neutrophil- or monocyte-endothelial cell interactions under defined laminar flow were studied in a parallel plate flow chamber as previously described.^{13,21} Briefly, confluent HUVEC monolayers were treated for 24 hours with HUVEC culture media alone or media containing 25 ng/ml TNF- α , 100 U/ml IFN- γ , or the combination of both IFN- γ and TNF- α . Pilot studies showed that this concentration of IFN- γ alone gave optimal induction of class I and ICAM-1 without loss of cell numbers or gap formation in HUVEC monolayers. Expression of E-selectin or up-regulation of VCAM-1 was not observed at this dose. Leukocytes were suspended to 0.5×10^6 cells/ml in DPBS containing 0.1% (v/v) human serum albumin, 0.75 mmol/L Ca^{2+} and Mg^{2+} , pH 7.4, and incubated for 10 minutes at 4°C with various mAbs. HUVEC monolayers were also incubated with appropriate test or control mAbs for 30 minutes at 37°C and then placed in the flow chamber. Neutrophils or monocytes were drawn through the chamber at a constant rate of 0.5 ml/min (estimated shear stress, 1.0 dyne/cm²). Leukocyte adhesion and transmigration were determined after 8 to 10 minutes of perfusion by analysis of four to six high-power ($\times 40$ for monocyte adhesion, $\times 20$ for neutrophil adhesion, and $\times 60$ for transmigration of both cell types) fields from videotape as detailed previously.^{13,17}

Static Transwell Assay

HUVECs (subculture 1) were removed with trypsin/versene and plated onto 0.1% gelatin-coated Costar transwells (3- μm pore size, 6.5-mm diameter), at 2.5×10^4 cells/well. After culture for 3 to 4 days, medium in upper and lower chambers was replaced with cytokines or with control medium for a further 24 hours. Neutrophils were added to the upper chamber at 2.5×10^5 per well, in

M199 medium. Neutrophils were allowed to transmigrate for 90 minutes, and then the upper chamber was removed to stop the assay. Transmigrated cells in the bottom chamber were counted using an electronic cell counter (model Z2; Beckman Coulter Electronics, Hialeah, FL). Each condition was performed in quadruplicate.

Flow Cytometry

HUVEC monolayers in M199 in 10% fetal calf serum alone or with cytokines were washed twice with HBSS⁻ and nonenzymatically harvested (5 mmol/L ethylenediaminetetraacetic acid in HBSS⁻ for 5 to 10 minutes at 37°C). A unicellular suspension of HUVECs was incubated with primary mAb for 30 minutes on ice, washed twice with RPMI/5% fetal bovine serum, and then primary mAb detected with a fluorescein isothiocyanate (FITC)-labeled secondary goat F(ab')₂ anti-mouse Ab (1:60 dilution; Caltag Laboratories, Burlingame, CA).²² The stained cells were washed twice and fixed in 1% formaldehyde-phosphate-buffered saline (PBS). A nonbinding murine mAb K16/16 primary control was used as a control. Fluorescence of 10^4 cells was detected using a Becton-Dickinson FACS Calibur flow cytometer (San Jose, CA).

Indirect Immunofluorescence Analysis of HUVEC Junction Proteins

HUVEC monolayers were cultured for 24 hours in M199 10% fetal calf serum alone or containing TNF- α , IFN- γ , both TNF- α and IFN- γ . Monolayers were washed gently three times in PBS and fixed in 10% neutral-buffered formalin (Sigma) at room temperature for 8 minutes and then rinsed three times in cold PBS.¹⁴ Fixed HUVEC coverslips were blocked with 10 mmol/L Tris-buffered saline (TBS) containing 0.1 mg/ml salmon sperm DNA, 1% horse serum, and 1% goat serum (block buffer) for 20 minutes at 37°C¹³ to reduce nonspecific binding of primary mAb. Monolayers were then incubated with primary mAb (each at 10 $\mu\text{g/ml}$) for 30 minutes at 37°C, rinsed three times in TBS, incubated with goat F(ab') anti-mouse IgG Cy-3 conjugate [1/75 dilution (v/v) in block buffer; Caltag Laboratories], washed twice in TBS, once in dH₂O and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images of stained monolayers were captured using a laser-scanning confocal microscope (BioRad MRC-1024/2P interfaced with a Zeiss Axiovert S100 microscope equipped with $\times 63$ water immersion objective).¹³ The laser power and image capture settings were optimized separately for each mAb using the media control-stained coverslip and these settings were maintained while collecting subsequent images of monolayers treated with various cytokines or the combination of cytokines. Serial 0.5- μm sections were taken routinely in the z direction and individual z-series were subsequently collapsed and manipulated using confocal Assistant (BioRad, Richmond, CA) and NIH Image 1.62 software. The composite images (each representing a collapsed image) in Figure 1 were assembled in exactly the same way using commercial software (Adobe Photoshop v5.1

Figure 1. Confocal laser-scanning immunofluorescence microscopy and quantitative image analysis of 24-hour cytokine-treated HUVEC monolayers stained for lateral junction proteins. **A:** Confluent HUVEC monolayers were treated with media alone or activated with IFN- γ (100 U/ml), TNF- α (25 ng/ml), or the combination of IFN- γ (100 U/ml) plus TNF- α (25 ng/ml) for 24 hours and then stained for JAM (mAb 2A9, **column 1**), PECAM-1 (mAb hec7, **column 2**), or VE-cadherin (TEA1/31, **column 3**) as described in Materials and Methods. For each mAb, images were obtained using laser-scanning confocal microscopy as detailed in Materials and Methods and exposures were optimized using media-treated cells. These settings were then held constant for the collection of images of HUVECs treated with various cytokines or combinations. Each image represents a collapsed z-series. By a $\times 20$ phase contrast objective, endothelial monolayers from each treatment remained intact and confluent (**vertical column** labeled "phase"). Results are representative of three to five separate experiments. The image labeled "second Ab" was stained with buffer only (no primary) followed by staining with Cy-3-conjugated secondary mAb. **B:** Quantitative fluorescence intensity of HUVECs treated with various cytokines or combinations. Images of HUVEC monolayers stained for surface Ag shown above were examined for surface immunofluorescence using MetaMorph software as detailed in Materials and Methods. The fluorescence intensity of a region (15×512 pixels) was marked by **open arrowheads** on each panel and then plotted as a histogram with the y axis representing the fluorescence intensity and the x axis representing distance (pixels). The peaks in fluorescence intensity for JAM, PECAM-1, and VE-cadherin represent the regions of intercellular junctions, and the troughs indicate the nonjunctional areas of staining. Each plot is representative of several separate measurements from two different experiments. The **dashed line** is media treatment and the **solid line** is TNF- α + IFN- γ treatment.

followed by Powerpoint, Microsoft Corp.). Phase contrast images were obtained using a Nikon microscope equipped with a $\times 20$ phase contrast objective and digitally recorded using a cooled charge-coupled device camera (SenSys, Photometrics) as detailed.¹³

Image Analysis of JAM, PECAM-1, and VE-Cadherin Surface Fluorescence

Fluorescence intensity of Ag was assessed using MetaMorph Imaging system (version 4.0, Universal Imaging Corp., Downingtown, PA). Briefly, using the line tool in MetaMorph, a 15×512 pixel region ($2.4 \text{ pixels} = 1 \text{ } \mu\text{m}$; $6.25 \times 210 \text{ } \mu\text{m}$) of interest was selected in each of the unenhanced collapsed images of VE-cadherin, PECAM-1, or JAM. The intensity was measured and the data plotted in MS-Excel, with the x axis depicting distance in pixels and the y axis the fluorescence intensity.

Endothelial Cell Paracellular Permeability Assay

HUVECs were plated onto gelatin-coated Costar transwells (0.4- μ m pore size, 6.5-mm diameter polycarbonate filters) at 2.5×10^4 cells/well. After culture for 4 days, medium in upper and lower chambers was replaced with cytokines or with control medium for a further 24 hours.

To measure permeability, medium in the upper chamber was replaced with HBSS⁺ without phenol red containing fluorescein-labeled dextran (FITC-dextran, 0.5 mg/ml, 70 kd, anionic; Molecular Probes, Eugene, OR). The size of FITC-dextran approximates that of human albumin, both of which have been used in similar paracellular permeability assays systems.²³ The lower chamber was replaced with HBSS⁺, and HUVECs incubated at 37°C for 1 hour. The inserts were then removed and the FITC fluorescence in the bottom chamber was read using a fluorescent plate reader (CytoFluor II, PerSeptive Biosystems). Each assay was performed in triplicate. In four experiments, after the permeability assay representative monolayers were stained using a modified Wright's Stain (Leukostat kit; Fisher Scientific, Pittsburgh, PA) and examined microscopically to ensure that monolayers were confluent and intact. Control experiments showed an increase in paracellular permeability with thrombin (data not shown).

Statistical Analyses

Statistical analyses by unpaired t -test was performed using Microsoft Excel 5.0 (Microsoft, Richmond, WA) and were considered statistically significant at $P \leq 0.05$.

Results

The Combination of TNF- α plus IFN- γ Induces Redistribution of JAM and PECAM-1 from Endothelial Cell Lateral Junctions

As reported by others,^{5,8,12} HUVECs maintained under static culture conditions in the absence of exogenous inflammatory cytokines exhibit an enriched lateral junction-staining pattern for PECAM-1 and VE-cadherin. JAM staining by four different mAbs revealed a more even distribution on the cell surface and most cells exhibited moderate enrichment at lateral junctions as compared to VE-cadherin (Figure 1a), which is similar to previous reports with human endothelium⁸ and a murine endothelial cell line.¹² Consistent with other reports,^{5,12} TNF- α or IFN- γ alone did not significantly alter this pattern of molecule localization when compared to media (Figure 1a; top row, panels labeled "media" versus second and third row panels, labeled "TNF" and "IFN," respectively). Experiments using higher concentrations of IFN- γ (200 to 1500 U/ml) with the same concentration of TNF- α (25 ng/ml) caused loss of endothelial cells during the 24-hour time course suggesting toxicity. Using the combination of TNF- α (25 ng/ml) plus 100 U/ml IFN- γ , however, caused a decrease in overall JAM and PECAM-1, but little change in VE-cadherin surface expression (Figure 1a, bottom). The reduction was similar throughout the z axis dimension indicating the loss was not because of sequestration in the basolateral or apical surfaces. There was no cell loss as demonstrated by the phase contrast photomicrographs (vertical panels labeled "phase").

The intensity of both JAM and PECAM-1 was reduced at cell-to-cell junctions as well as on the nonjunctional regions as assessed using quantitative image analysis of fluorescence. A 15×512 pixel ($6.25 \times 210 \mu\text{m}$) region of interest was selected for each image, indicated by open arrowheads in the images of Figure 1a. The intensity of fluorescence was determined using commercial software and then plotted as a histogram in Figure 1b (dotted line, media; solid line, TNF- α + IFN- γ). As a reference point, the peaks of fluorescence intensity for JAM (Figure 1b, top) and PECAM-1 (Figure 1b, middle) after treatment with media correspond to lateral junction regions, whereas the valleys correspond to nonjunctional regions. Dual cytokine treatment significantly blunted the peaks of fluorescence intensities that correspond to junctional staining for PECAM-1 and JAM, but not VE-cadherin. Reiterative analysis of several regions of the same image gave a similar pattern of fluorescence tracings and revealed a pronounced decrease in JAM and PECAM-1 intensity after dual cytokine treatment. In addition, the morphology of endothelial cells appeared elongated as assessed by indirect immunofluorescence laser-scanning confocal and phase-contrast microscopy (Figure 1a) as reported previously.^{5,24}

To corroborate the quantitative fluorescence image analysis results, the total surface expression of these junctional proteins was quantified by indirect immunofluorescence and flow cytometry. In three separate ex-

periments, dual cytokine treatment caused a significant decrease in both JAM ($43 \pm 2\%$ decrease, $P < 0.05$) and PECAM-1 ($58 \pm 4\%$ decrease; $P < 0.05$) surface expression (Figure 2 shows a representative experiment). Overall, however, the antigens were detected at both 4- and 24-hour time points (only 24 hours presented for comparison to Ozaki and colleagues¹²). The total surface expression of VE cadherin did not change significantly under any conditions. In addition p96, a 96-kd TGF- β binding protein, was not significantly reduced by any single or combination of cytokine treatments, which is consistent with earlier *in vitro* experiments.¹⁹ TNF- α alone did not alter the total surface expression of p96, VE-cadherin, PECAM-1, or JAM. We also confirmed that the dual cytokine treatment led to a synergistic increase in class I (HLA-A,B) molecule expression as compared to either cytokine alone as previously noted (data not shown).¹⁹ Taken together, the results suggest that at the concentrations tested here, the cytokines alone or in combination had no direct cytotoxic effect on HUVEC monolayers but caused a dramatic reduction and redistribution of JAM and PECAM-1 away from junctions.

TNF- α plus IFN- γ Treatment Up-Regulate Leukocyte Adhesion and Transmigration under Flow

We next tested directly whether redistribution of lateral junction proteins by cytokines reduces blood neutrophil and monocyte transmigration, as was proposed recently.^{5,12} IFN- γ alone did not induce an increase in neutrophil adhesion or transmigration under flow (Figure 3) although a small, but statistically significant, increase in monocyte adhesion and transmigration was observed (Figure 4). This is consistent with the observation that IFN- γ alone does not trigger induction of molecules such as E- or P-selectin or VCAM-1 by HUVECs^{2,3} that can initiate initial attachment and/or rolling of neutrophils under flow (data not shown). In contrast, TNF- α alone or TNF- α plus IFN- γ caused an equivalent increase in both neutrophil and monocyte adhesion and subsequent transmigration (Figures 3 and 4). By live-time video microscopy, after 6 minutes of perfusion, many leukocytes had attached, rolled a short distance (monocytes) or variable distances (neutrophils), and arrested on the apical endothelial cell surface. Shortly after arrest (56 ± 23 seconds for TNF- α , $n = 21$; 57 ± 12 seconds for TNF- α + IFN- γ , $n = 21$) a significant percentage of these adherent neutrophils had transmigrated across the endothelial monolayer. There were no obvious differences in the time course of neutrophil or monocyte transmigration for HUVEC monolayers treated with TNF- α alone or with the combination of cytokines.

Monocyte Transmigration Is Still Dependent on PECAM-1

To investigate whether monocyte transmigration was still dependent on PECAM-1 despite its redistribution away

Figure 2. Effects of cytokines on surface expression of endothelial cell PECAM-1, JAM, and VE-cadherin. Confluent HUVECs were washed once with culture media and incubated with culture media containing IFN- γ (100 U/ml), TNF- α (25 ng/ml), or IFN- γ (100 U/ml) plus TNF- α (25 ng/ml) or media alone for 24 hours. HUVECs were then subjected to indirect immunofluorescence and flow cytometry as detailed in Materials and Methods. The y axis is cell number and the x axis is mean channel fluorescence (log scale). The number in the **top right** corner of each panel is the mean channel fluorescence intensity for one experiment and are representative of three separate experiments that showed essentially the same relative loss in PECAM-1 and JAM expression.

from lateral junctions and overall decreased level of expression by treatment with TNF- α plus IFN- γ , the endothelial monolayers were treated with function-blocking PECAM-1 mAbs before assay and monocyte transmigration was assessed in the flow assay. Monocytes were examined because this leukocyte type exhibited significant dependency on PECAM-1 for transmigration under flow.^{13,18} Inhibition of PECAM-1 with mAb had no effect on monocyte adhesion, but reduced transmigration by 66% as compared to isotype-matched control binding Hu8/4 mAb (nonblocking CONT. VCAM-1) or binding control mAb W6/32 (MHC class I; Figure 5). This result indicated that reduction of PECAM-1 from lateral junctions does not diminish its role in monocyte transmigration. As controls, blockade of both VCAM-1 and ICAM-1 or monocyte integrins CD18 and VLA-4 ($\beta 2 + \alpha 4$) significantly reduced adhesion and transmigration of adherent cells. If the results are normalized (as percent transmigration) to take into account the reduction in adhesion, the blockade of VCAM-1/ICAM-1 or $\beta 1/\beta 2$ -integrins still

significantly reduced monocyte transmigration (Figure 5, bottom).

We also assessed whether a panel of mAbs to human JAM could block neutrophil or monocyte adhesion or transmigration in our flow assay. Two recently reported mAbs to human JAM, J3 and J10, block recovery of barrier function and tight junction reassembly in epithelial monolayers after disruption by transient Ca^{2+} depletion.¹⁴ A third mAb to JAM (mAb F11) has been shown when immobilized to trigger platelet shape change and adhesion.¹⁵ These published reports suggest that the epitopes recognized by the different mAbs may be important in JAM-mediated cell-cell interactions. We next examined the ability of these mAbs to alter leukocyte interactions with 24-hour TNF- α -activated HUVEC monolayers under conditions that do not alter surface expression or localization of JAM. Pretreatment of both HUVECs and leukocytes with mAbs J10, J3, or F11 had no inhibitory effect on neutrophil (Figure 6) and monocyte (data not shown) adhesion or the percent of transmigration. As

Figure 3. Neutrophil adhesion to cytokine-activated HUVEC monolayers under flow conditions *in vitro*. Confluent HUVEC monolayers were cultured with media alone or media containing IFN- γ (100 U/ml), TNF- α (25 ng/ml), or IFN- γ (100 U/ml) plus TNF- α (25 ng/ml) for 24 hours, rinsed carefully, and inserted into a parallel plate flow apparatus for adhesion assay as detailed in Methods and Materials. Neutrophils (0.5×10^6 /ml in DPBS⁺ containing 0.1% human serum albumin) were drawn through the chamber at 0.52 ml/min (estimated shear stress = 1 dyne/cm²). Adhesion was determined after 4 to 6 minutes of flow using a $\times 20$ phase objective and neutrophil transmigration was assessed after 6 to 8 minutes of flow using a $\times 60$ phase contrast objective as previously detailed.¹³ *, Value statistically significant from media treatment. Data are means $\pm$ SD of four separate experiments performed in duplicate.

controls, mAb to CD18 reduced neutrophil adhesion and the percent transmigration under flow (Figure 6) and the combination of VCAM-1 and ICAM-1 also blocked adhesion and reduced the percent transmigration. A nonfunction blocking control VCAM-1 mAb (Hu8/4, cont. VCAM)

Figure 4. Monocyte adhesion to cytokine-activated HUVEC monolayers under flow conditions *in vitro*. Confluent HUVEC monolayers were cultured with media alone or media containing IFN- γ (100 U/ml), TNF- α (25 ng/ml), or IFN- γ (100 U/ml) plus TNF- α (25 ng/ml) for 24 hours, rinsed carefully, and inserted into a parallel plate flow apparatus to determine adhesion and transmigration. Data are means $\pm$ SD from four separate experiments. *, $P < 0.05$ versus media alone.

had no effect on adhesion or transmigration of either leukocyte type.

Effects of Cytokines on HUVEC Monolayer Paracellular Permeability and Leukocyte Transmigration under Static Conditions

Another possible explanation for the reduction in neutrophil transmigration across dual cytokine-treated HUVECs

Figure 5. PECAM-1 mAb or anti-VLA4/ β -2 integrins mAb reduces monocyte transmigration across IFN- γ - and TNF- α -activated HUVEC monolayers under flow conditions *in vitro*. Confluent HUVEC monolayers were cultured with media alone or media containing IFN- γ (100 U/ml) plus TNF- α (25 ng/ml) for 24 hours. Monolayers and monocytes were pretreated (20 minutes) with anti-PECAM-1 mAb (Hec-7), anti-VCAM-1, and ICAM-1 (E1/6 + Hu5/3), α 4 and β 2-integrins (HP2/1 + TS1/18), anti-class I (W6/32), or CONT. VCAM-1 (nonblocking Hu8/4). HUVEC monolayers were then carefully inserted into a parallel plate flow apparatus for adhesion assay as detailed in Figure 3. The percent transmigration equals the number of transmigrated neutrophils divided by the number of adherent and number of transmigrated neutrophils $\times$ 100. *, Value statistically significant ($P < 0.05$) from cytokine-activated HUVECs with media or control mAb (control VCAM-1 HU8/4) or media treatment. Data are means $\pm$ SD of four separate experiments performed in duplicate.

cultured in transwells under static conditions is an increase in barrier function under these conditions leading to decreased movement of leukocytes across the monolayer. This possibility was assessed by measuring the paracellular permeability. Based on seven separate experiments, 24-hour IFN- γ treatment alone, or in combination with TNF- α , caused a consistent and nearly twofold

Figure 6. Effects of JAM mAb inhibition on neutrophil adhesive interactions with 24 hours TNF- α -activated HUVEC monolayers under flow. Confluent HUVEC monolayers were activated with TNF- α for 24 hours, incubated with various mAbs, and inserted into a parallel plate flow apparatus for measurement of adhesion and transmigration. Neutrophils were treated with various mAbs (10 minutes at 4°C) and drawn through the chamber. Adhesion and transmigration were determined as in Figure 3. The percent transmigration was calculated as follows: number of transmigrated neutrophils divided by the number of adherent plus number of transmigrated neutrophils $\times$ 100. Data are means $\pm$ SD from four separate experiments. *, $P < 0.05$ indicates a value significantly lower than class I mAb, cont. VCAM-1, or cytokines plus media treatments.

increase in paracellular permeability in HUVECs cultured in a transwell system, indicating a loss rather than a gain in paracellular barrier function (Figure 7). TNF- α alone did not significantly influence endothelial cell permeability at 24 hours or at 4 hours (data not shown). These results indicate that increased paracellular permeability does not account for the previously reported negative regulation of neutrophil transmigration by cytokines.^{5,12}

Given the barrier function results, we attempted to confirm the reduction in neutrophil transmigration across cytokine-treated HUVECs in a transwell assay under static conditions. The number of transmigrated neutrophils increased 3.7-fold with TNF- α , 2.2-fold with IFN- γ , and 2.2-fold with the combination of TNF- α and IFN- γ as compared to media treatment alone (Table 1). The trend and number of migrated cells with each cytokine treatment were similar to the results previously reported.⁵

Figure 7. Cytokine activation increases endothelial cell paracellular permeability. HUVEC monolayers were activated for 24 hours with various cytokines. Cytokines were removed by washing and monolayers were incubated for 60 minutes with FITC-dextran 70 kd. The amount of tracer in the bottom well of the insert was measured as detailed in Materials and Methods. Data are means $\pm$ SD from seven separate experiments. **Asterisks**, $P < 0.005$ as compared to media control, using Student's *t*-test.

Thus, our results indicate that significant transmigration still occurs under static conditions after various cytokine treatments. It is of interest to note that in a flow assay, IFN- γ treatment does not increase neutrophil adhesion and minimally increased monocyte adhesion, whereas under static conditions there is a large increase in adhesion and transmigration.

Discussion

In this study we confirm that the combination of inflammatory cytokines TNF- α and IFN- γ causes decreased localization of JAM and PECAM-1 at endothelial cell lateral junctions. However, we find that loss and/or redistribution of these proteins did not reduce blood neutrophil or monocyte adhesion or transmigration in contradiction to previous reports.^{5,12} The major difference between the current study and previous ones^{5,12} is that we have per-

formed adhesion assays under conditions that mimic blood flow in small vessels *in vivo*. Thus, analysis by live time videomicroscopy using an *in vitro* flow assay showed no change in either the percentage of adherent leukocytes (monocytes or neutrophils) that transmigrate or the time required for migration across dual cytokine-activated HUVECs as compared to monolayers treated with TNF- α alone. Moreover, even though PECAM-1 was less abundant at lateral junctions after treatment with the combination of cytokines, mAb blockade still inhibited 66% of monocyte transmigration, which is similar to the level of inhibition for TNF- α -activated endothelium (data not shown and our previous work^{13,17}). These results suggest that PECAM-1 participates in transmigration and that its reduced expression at the junction after dual cytokine treatment does not limit its role for leukocyte transmigration in this system. One can speculate that PECAM-1 adequately performs a signaling function²⁵ even with reduced levels after dual cytokine treatment. The results under flow conditions here are consistent with *in vivo* studies that have found that TNF- α and IFN- γ cytokines locally activated the endothelium, induced leukocyte accumulation, and increased endothelial cell permeability.¹ We conclude that redistribution of PECAM-1 and JAM in cultured endothelial monolayers does not negatively regulate leukocyte transmigration under conditions of flow *in vitro*.

We explored other possible explanations for the observed differences in transmigration. In particular, we focused on whether alterations in HUVEC barrier function could explain the differences in leukocyte transmigration under static conditions. The basic premise was that dual cytokine treatment resulted in a decreased paracellular permeability (ie, enhanced barrier function) that correlated with the reduced transmigration in static transwell assays. In the transwell permeability assay, however, IFN- γ alone, or in combination with TNF- α , increased rather than decreased permeability, whereas TNF alone had no effect (Figure 7). The increase in permeability is consistent with previous *in vivo*¹ and *in vitro* studies.²⁶ In addition, we also repeated transmigration studies under static conditions using a transwell assay and found similar results as Rival and colleagues⁵ (Table 1). Our reagents and cell types, therefore, afford comparable results. We conclude that assay under defined flow conditions, when compared to static conditions, more accurately reflect leukocyte adhesive interactions with cytokine-treated endothelium and more closely mimic the results from *in vivo* experiments.¹ Furthermore, changes in endothelial cell paracellular permeability for molecules such as FITC-dextran, do not necessarily parallel changes in rate of leukocyte transmigration when assayed under flow.

One explanation for the differences in transmigration between flow and static assays may reside in the sensitivity of these two systems to the cytokine-induced alterations in PECAM-1 and JAM localization. It may be that a threshold level of JAM, PECAM-1, or other molecules at junctions, are required for initiating transmigration and that under flow this threshold is met, whereas static conditions do not meet or exceed the threshold. In this case fewer migration events occur under static conditions.

Table 1. Effect of Cytokine Treatment on Neutrophil Transmigration Under Static Conditions

Treatment	Transmigrated neutrophils/well* ($\times 10^3$)	Fold-increase over media control
Media control	23.4 $\pm$ 4.4	1.0
TNF- α	87.5 $\pm$ 14.1 [†]	3.7
IFN- γ	50.9 $\pm$ 2.1 [†]	2.2
TNF- α + IFN- γ	52.4 $\pm$ 5.0 [†]	2.2

*Data are means $\pm$ SD of quadruplicate wells. Representative of four other separate experiments.

[†] $P < 0.05$ as compared to media control, using Student's *t*-test.

Another possibility, which is not mutually exclusive, is that fluid shear stress imposes a force or signal in endothelium, or leukocyte, or both cell types, that in concert with chemokines or adhesion molecules enables rapid transmigration across dual cytokine-activated monolayers, which may not be appreciated in static assays. The effects of fluid shear stress on endothelial cells has been widely documented.^{27,28} For example, fluid shear stress can trigger rapid responses (NOS induction, PGI₂ production, rise in intracellular Ca²⁺) as well as long-term effects in endothelium (transcription of genes containing SSRE motif,²⁸ reorganization of cell cytoskeleton and cell shape). Leukocyte adhesiveness has been shown to be dependent on shear stress *in vivo*.²⁹ More recently, leukocyte transendothelial migration has been shown to be promoted by shear stress,³⁰ suggesting that flow-based assays may be a better technique than static assays for measuring transmigration.

JAM localizes to lateral junctions in endothelium and epithelium,^{14,31,32} and has previously been implicated in leukocyte recruitment in *in vivo* murine models of inflammation.^{8,10} The human homologue of JAM is also expressed at endothelial and epithelial junctions, as well as on hematopoietic cells of all lineages,^{9,14} and was shown to regulate tight junction resealing in human T84 epithelial monolayers.¹⁴ Liu and colleagues¹⁴ reported that two anti-JAM mAbs, J3 and J10, and a polyclonal antiserum had no inhibitory effect on fMLP-stimulated neutrophil transepithelial migration. The role of JAM in a human endothelial cell system has not been addressed previously under flow conditions. Here, multiple murine mAbs had no inhibitory effect on neutrophil or monocyte initial attachment, rolling, stable adhesion, or transmigration across 24-hour TNF- α -treated HUVECs (Figure 6 and data not shown). The lack of effect by mAbs J3, J10, and F11 could be because of recognition of an epitope not involved in leukocyte adhesive and transmigration functions, and/or that human and murine JAM have differing physiological roles in inflammation. A recent study has suggested how JAM may function in leukocyte recruitment.³³ Using murine recombinant soluble JAM, these authors suggest this molecule forms noncovalent dimers and that mAb BV11, which blocks leukocyte recruitment in *in vivo* models of inflammation, preferentially recognizes JAM dimers and blocks dimer homophilic adhesive interactions. The authors speculate that mAb BV11 blocks recruitment by interfering with a homophilic adhesive interaction between leukocyte JAM and endothelial cell JAM.³³

The mechanism(s) underlying the change in PECAM-1 and JAM localization by cytokine treatments are controversial.^{5,12,24} The change in PECAM-1 localization using multiple quantitative strategies has been explained by a decrease in total synthesis with no redistribution⁵ or alternatively, by a redistribution without a reduction in total amount of protein.^{12,24} In contrast to PECAM-1, the only other report of cytokine effects on JAM concluded that JAM was redistributed from lateral junctions without a loss in total amount.¹² The current results using both image analysis of stained monolayers and indirect immunofluorescence followed by flow cytometry favor a de-

crease in the total surface expression of JAM and PECAM-1 and marked redistribution away from lateral junctions. It also seems that other newly described molecules, such as CD99, which localizes to endothelial cell lateral junctions and participates in leukocyte transmigration³⁴ or claudin-5,³⁵ which localizes to lateral junctions and is implicated in tight junction formation, may be involved, either directly or indirectly, in controlling leukocyte infiltration. Future studies examining the effects of cytokines on these molecules will provide insight into the relationship of junction localization and leukocyte infiltration.

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