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Vascular Endothelial-Junctional Adhesion Molecule (VE-JAM)/JAM 2 Interacts with T, NK, and Dendritic Cells Through JAM 3

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Screening expressed sequence tag databases for endothelial-specific homologs to human junctional adhesion molecule (JAM) and A33-Ag, we identified a protein of 298 aa that represents the recently described vascular endothelial-JAM (VE-JAM)/JAM 2. We confirmed VE-JAM/JAM 2 expression to be restricted to the high endothelial venule of tonsil and lymph nodes, and we further expanded the localization to the endothelium of arterioles in and around inflammatory and tumor foci. In our functional characterizations of VE-JAM/JAM 2, we discovered that it can function as an adhesive ligand for the T cell line J45 and can interact with GM-CSF/IL-4-derived peripheral blood dendritic cells, circulating CD56+ NK cells, circulating CD56+CD3+ NK/T cells, and circulating CD56+CD3−CD8+ cytolytic T cells. In the course of our studies, we also isolated and characterized the functional VE-JAM/JAM 2 receptor, which, upon cloning, turned out to be a submitted sequence representing JAM 3 (accession number NP_113658). With these understandings, we have characterized a protein-interacting pair that can be important in the role of T, NK, and dendritic cell trafficking and inflammation. The Journal of Immunology, 2002, 168: 1618–1626.

The recent discovery and description of junctional adhesion molecule (JAM)2 and A33-Ag introduced a new subfamily of Ig-like adhesion molecules to the study of epithelial biology and tight junction regulations. These reports also suggested that molecules similar to JAM and A33-Ag might exist in an endothelial-specific fashion, playing an important role in endothelial biology and lymphocyte trafficking into and out of the specific endothelial environments termed high endothelial venules (HEV). In search of a molecule, we identified an endothelial-specific JAM homolog that proved to be the previously reported vascular endothelia-JAM (VE-JAM)1 (1) or JAM 2 (2), termed herein as VE-JAM/JAM 2. VE-JAM/JAM 2 is described in this work, as previously reported (1, 2). It is expressed in a variety of fetal vascular endothelia, the HEV of the adult tonsil and lymph nodes. We have extended VE-JAM/JAM 2 localization by observing a preferential expression of VE-JAM/JAM 2 mRNA in the endothelium of arterioles in and around tumors and sites of inflammation. Furthermore, VE-JAM/JAM 2 mRNA was also detected in the spermatogenic cells of the epithelium in testicular seminiferous tubules as well as the intermediate trophoblasts of the placenta. VE-JAM/JAM 2 was also confirmed as an adhesion molecule for T cell lines (2) and observed to interact with a very specific subpopulation of circulating lymphocytes, namely the CD56+ NK cells, the CD56+CD3+ NK/T cells, the CD56+CD8+CD3+ cytolytic T cells, and peripheral blood, GM-CSF/IL-4-derived dendritic cells (PBDC). Also reported in this work are the cloning and identification of the VE-JAM/JAM 2 counterligand, which was identified as JAM 3 (accession number NP_113658), making VE-JAM/JAM 2 and JAM 3 the first known interacting pair in the A33/JAM family of adhesion molecules.

Materials and Methods

Cloning and construction of Fc fusion proteins

Human VE-JAM/JAM 2 and JAM 3 cDNA were isolated from a human colonic cDNA library by colony hybridization. Human IgG1 Fc fusion protein (immunoadhesins) of VE-JAM/JAM 2 (VE-JAM/JAM 2.Fc) or JAM 3 (JAM 3.Fc) was prepared, as previously described (3), and purified over a protein A column (Amersham Pharmacia Biotech, Piscataway, NJ). Identity of the purified protein was verified by N-terminal sequence analysis.

Expression cloning of JAM 3

Identification of JAM 3 was done by transiently transfecting pooled cDNA libraries encoding secreted and transmembrane proteins into COS cells grown on glass chamber slides. Twenty-four hours after transfection, VE-JAM/JAM 2.Fc was added (0.5 μg/ml) and incubated for 30 min. VE-JAM/JAM 2.Fc binding was determined. Positives were processed as previously described (4).

Ab generation

BALB/c females were immunized and boosted with 10 μg of VE-JAM/JAM 2.Fc or 8×His-tagged JAM 3 via footpad injections, as previously described (5). Single clones were screened against VE-JAM/JAM 2.Fc or 8×His-tagged JAM 3. Selected clones were tested for cross-reactivity against A33/JAM family members and human IgG Fc. Clones were titrated out to single cell densities and rescreened. Clone 12D10.2F9 (IgG1 κ) was discovered to be selectively reactive to VE-JAM/JAM 2 and not to JAM or JAM 3. Clone MoJIR1 (IgG2b) was found to be selectively reactive to JAM 3 and not to JAM or VE-JAM/JAM 2. Both clones were isolated and used for ascites fluid generation (6). Abs were purified over a protein G column (Amersham Pharmacia Biotech); protein concentration was determined using the Pierce BCA reagent (Pierce, Rockford, IL).
Expression of VE-JAM/JAM 2 in CHO cells

VE-JAM/JAM 2 cDNA was amplified by PCR from a human colon cDNA library (Clontech Laboratories, Palo Alto, CA) using primers specific for the 5’ and 3’ ends of the coding sequence. The amplified fragment was gel purified, digested (BanHI and HindIII), extracted in phenol–chloroform–isoamyl alcohol (Life Technologies, Gaithersburg, MD), lyophilized, ligated into pBluescript II vector (pSD5.muJAM), transfected into Chinese hamster ovary (CHO) cells, and selected, as previously described (7). Stable clones were screened for 12D10.2F9 reactivity; one clone, CHO-JI, found to have the highest expression, was used for characterization and further studies.

In situ hybridization

PCR primers (upper, 5′-GGGAAAGTTGCGAGGAGGAG, and lower, 5′-CCCAAAGCCACAAGGAAATC) were designed to amplify a 776-bp fragment of VE-JAM/JAM 2. Primers included T7 or T3 RNA polymerase initiation sites to allow for in vitro transcription of sense or antisense probes, respectively, from the amplified products. Normal human tissues included tonsil, lymph node, spleen, kidney, urinary bladder, lung, heart, aorta, coronary artery, liver, gall bladder, prostate, stomach, small intestine, colon, pancreas, thyroid gland, skin, adrenal gland, placenta, uterus, ovary, testis, retina, brain (cerebellum, brainstem, cerebral cortex), and human fetal tissues (12–16 weeks gestation). Tissues with chronic inflammatory disease included lungs with chronic asthma, chronic bronchopneumonia, chronic bronchitis/chronic obstructive pulmonary disease, kidneys with chronic lymphocytic interstitial nephritis, and livers with chronic inflammation and cirrhosis due to chronic hepatitis C infection, autoimmune hepatitis, or alcoholic cirrhosis. Primary human neoplasms were benign or malignant liver carcinoma, pulmonary adenocarcinoma, prostatic adenocarcinoma, and colonic adenocarcinoma. Tissues were fixed (4% formalin), paraffin-embedded, sectioned (3–5 μm thick), deparaffinized, deproteinized (20 μg/ml) with proteinase K (15 min at 37°C), and processed for in situ hybridization (8). Probes were 33-μP-labeled, hybridized overnight (55°C), washed (0.1× SSC for 2 h at 55°C), dipped in NBT2 nuclear track emulsion (Eastman Kodak, Rochester, NY), exposed (4 wk at 4°C), and developed and counterstained with H&E.

Cell culture

J45 and Ramos cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and grown in conditions defined by ATCC.

Purification of peripheral blood cells

Blood was obtained from healthy adult volunteers by venous puncture and separated using Ficoll-Paque PLUS (Amersham Pharmacia Biotech) per the manufacturer’s instructions. PBMC were obtained from the interface, washed in cold PBS, lysed (with 0.2% NaCl for 30 s) and neutralized with 1.6% NaCl as needed, counted, and kept on ice at 5 × 10^6 cells/ml until use. By flow cytometric analysis, no contaminating platelets were observed in the purified PBMC fractions. Neutrophils were obtained from the pellet after lysis of contaminating RBCs. Neutrophils were washed in cold PBS, counted, and kept at 5 × 10^6 cells/ml until use. To isolate peripheral blood subsets, “untouched” MACS kits (Miltenyi Biotech, Auburn, CA) were used following the manufacturer’s instructions.

Protein conjugation

VE-JAM/JAM 2.Fc, human IgG1, or JAM 3.Fc were biotinylated with 200 μg of EZ-Link sulfo-NHS-LC-biotin (Pierce) per 1 mg of protein in PBS for 30 min at room temperature. Biotinylation was quenched with the addition of (final concentration) 200 mM Tris, pH 8, and incubated for 30 min at room temperature. Biotinylated proteins were then dialyzed extensively against PBS and concentrated to a concentration of 2 mg/ml with Centricon-10 microconcentrators (Millipore, Bedford, MA).

Alexa-488 (Molecular Probes, Eugene, OR) protein conjugation kit was used per the manufacturer’s instructions for the conjugation of Alexa-488 onto VE-JAM/JAM 2.Fc or human IgG1.

Isolation of VE-JAM/JAM 2-binding cells by magnetic sorting

Biotinylated VE-JAM/JAM 2.Fc or human IgG1 were incubated (1 h at 4°C) with PBMC (10^7 cells/ml) in SerF buffer (10% FBS (v/v); Life Technologies) plus 0.1% NaN₃ (w/v; Sigma-Aldrich, St. Louis, MO) in HBSS without calcium and magnesium containing EDTA instead of the normal HBSS (5). Cells were washed with SerF buffer, and resuspended at 80 μl/10^7 cells. Streptavidin magnetic beads (Miltenyi Biotech) were added to 20 μl/10^7 cells and incubated for 15 min at 4°C, washed with SerF buffer, resuspended at 500 μl/10^6 cells, and passed over a positive selecting MACS column (Miltenyi Biotech), per the manufacturer’s instructions. Positively selected cells were eluted per the manufacturer’s instructions, washed with SerF buffer, and analyzed by flow cytometry for surface CD Ags at 2 × 10^6 cells per condition. In all experiments conducted with this method, a large discrepancy was observed in the recovery of VE-JAM/JAM 2.Fc-binding cells vs human IgG1-binding cells. Whereas only ~25% of total applied cells could be recovered in the VE-JAM/JAM 2.Fc conditions, >40% of total applied cells were recovered in the human IgG1 conditions. This discrepancy could only be attributed to the function of the FcR, as the presence of excess nonbiotinylated human IgG1 cannot cross-compete biotinylated human IgG1, but can only establish a steady state binding of nonbiotinylated vs biotinylated human IgG1. For VE-JAM/JAM 2.Fc, the nonbiotinylated human IgG1 is competing for the FcR, leaving the VE-JAM/JAM 2 portion of the Fc-fusion protein free to interact with cell surface receptors. Because these data are presented as percentage of positively stained cells in a total of 2 × 10^6 cells collected per staining condition for flow cytometry.

Flow cytometry and FACS sorting

Cells for use in flow cytometric analysis were blocked for 30 min at 4°C with SerF buffer and stained with Abs to CD3, CD4, CD8, CD14, CD19, or CD56, conjugated to either FITC, PE, or CyChrome (BD Pharmingen, San Diego, CA). For sorting, cells were incubated (30 min at 4°C) with Alexa-488-conjugated human IgG1 or VE-JAM/JAM 2.Fc (10 μg/ml cells) in a modified SerF buffer (SerF buffer with 5 μg/ml anti-CD16 Ab 3G8 (BD Pharmingen) and 20 μg/ml human IgG1 (Calbiochem, San Diego, CA)), washed, and sorted on an Elite ESP (Beckman Coulter, Miami, FL). In these conditions, Alexa-488-conjugated human IgG1 was used as a negative control. For competition experiments, the competitor (20 μg/ml cells) was mixed with the cells for 20 min at room temperature in SerF buffer before Alexa-488-conjugated VE-JAM/JAM 2 or human IgG1 were introduced. The cells were then washed and analyzed by flow cytometry.

Adhesion/ELISA

For all assays, microtiter wells (NUNC Maxisorb 96-well plates; VWR, Scientific Products, Brisbane, CA) were coated with conditions at 50 μl/well (in HBSS), 10 μg/ml for 2 h at room temperature, unless otherwise noted. For adhesion assays, 50 μl of 10 μg/ml goat anti-human IgG1 Fc-specific Ab was first coated and blocked before the addition of conditions in binding/blocking buffer (BBB; HBSS containing 10% (v/v) FBS) for 1 h at room temperature before the addition of coating condition. Cells (5 × 10^5 cells/ml in BBB) were treated (10 min, 37°C, 5% CO₂) with 5 μg/ml 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF AM) (Molecular Probes, Eugene, OR) to allow adhesion to coated wells (2 × 10^5 cells/well in BBB) in 1 h at 37°C, 5% CO₂. Plates were read on a SpectraMax fluorescence plate reader (Molecular Devices, Sunnyvale, CA) for total applied fluorescence, gently washed three times (by aspiration with a 28-gauge needle), and read for total adherent fluorescence. Percentage of adherence was calculated using the following equation: ((total fluorescence of adherent)/total fluorescence of applied cells) × 100. Plates were incubated at 4°C > 3 h before reading. BCECF AM-labeled J45 cells. Values obtained from the blank wells (percentage of adherence) were subtracted from all experimental conditions to derive a final value. For ELISA, the plates were blocked after condition coating with BBB for 30 min at room temperature and incubated with binding conditions for 1 h at room temperature. For conditions requiring EDTA, a modified BBB (HBSS without calcium and magnesium containing EDTA instead of the normal HBSS) was used through the experiment. Plates were washed three times, incubated with 1 μg/ml streptavidin HRP (Pierce) for 30 min at room temperature, and assessed via color development using the tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and read on the Thermomax Microplate Reader (Molecular Devices).

Immunoprecipitation and Western blotting

For biotinylated conditions, cells were first washed in HBSS before being biotinylated (200 μg/10^6 cells) with sulfo-NHS-LC-biotin (30 min at 4°C). Cells were washed with TBS (30 min at 4°C) to quench the biotinylation.

Cells were lysed (10^6 cells/ml with lysis buffer (HBSS containing 1% Triton X-100 and 1 Complete-Mini EDTA free protease inhibitor tablet (Roche Biochemicals, Indianapolis, IN) per ml of lysis buffer) for 30 min at 4°C. Lysates were spun at 22,000 × g (1 h at 4°C) and 0.2 μm filtered. Lysates were precleared (2 h at 4°C) with 5 μl/10^6 cells of recombinant protein A beads (Amersham Pharmacia Biotech). Cleared lysates were 0.2 μm filtered and incubated (2 h at 4°C) with 5 μg/10^6 cells of either VE-
JAM/JAM 2.Fc or human IgG1, conjugated to protein A matrix using the ImmunoPure Protein A IgG Plus Orientation kit (Pierce). Beads were pelleted and washed with lysis buffer and denatured by the addition of 15 μl/10^6 cells of nonreducing SDS sample buffer (standard sample buffer with 2 mM iodoacetamide, but without DTT or 2-ME) and boiled for 3 min at 100°C.

Samples (at 15 μl/lane) were resolved on a 4–20% Bio-Rad Tris-HCl Ready Gel (Bio-Rad, Hercules, CA) and transferred onto 0.2-μm Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 100 mA for 2 h at 4°C. Blots were blocked for 1 h in Blotto (TBS containing 5% nonfat milk and 0.05% Tween 20; Bio-Rad). For biotinylated samples, HRP-conjugated streptavidin (Pierce) was used at 0.5 μg/ml for 30 min at room temperature. For nonbiotinylated samples, MaJIR1 was used at 10 μg/ml in Blotto and incubated for 1 h at 25°C before the application of 1 μg/ml HRP-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA) in Blotto for 30 min at room temperature. Blots were washed thoroughly with TTBS (TBS containing 0.05% Tween 20) and developed with the ECL Plus reagent (Amersham Pharmacia Biotech) before exposing onto Kodak BioMax ML film and development with Kodak M35A X-Omat Film Processor (Eastman Kodak).

Results

Cloning of the A33/JAM family proteins VE-JAM/JAM 2

Screening expressed sequence tag databases for homologies to JAM and A33-Ag, we identified a full-length cDNA that encodes alternative splice forms of 298 or 312 aa, differing in the C terminus. The shorter of the two forms is the recently described VE-JAM (1), or JAM-2 (2), termed in this study as VE-JAM/JAM 2. Similar to human JAM (huJAM) (9–11) and A33-Ag (12), VE-JAM/JAM 2 is predicted to possess two potential Ig-like loops in the extracellular domain: a short single-transmembrane domain and a short cytoplasmic tail (1, 2).

Localization of VE-JAM/JAM 2 on normal tissue

Expression of VE-JAM/JAM 2 mRNA was evaluated by in situ hybridization. In the evaluated human fetal tissues (E12–16 wk brain, spleen, bowel, and thyroid), VE-JAM/JAM 2 expression was predominantly endothelial. More specifically, VE-JAM/JAM 2 was present only in the vascular endothelium of small and large vessels (excluding capillaries), in mesenteric vessels, mural vessels of the bowel wall, and small vessels of the developing mesenteric lymph nodes and thyroid (data not shown). Expression was absent in spleen.

Expression of VE-JAM/JAM 2 mRNA in normal adult human tissue was restricted. Expression was found in the endothelium of HEV in tonsils and lymph nodes (data not shown) (1), the spermatogenic cells of the epithelium in the testicular seminiferous tubules (Fig. 1, I and J), and the intermediate trophoblasts of the placenta (data not shown).

Localization of VE-JAM/JAM 2 on inflamed tissue

The expression of VE-JAM/JAM 2 was more extensive in tissues with chronic inflammatory diseases. Lung tissues included lungs with chronic asthma, chronic bronchopneumonia, and chronic bronchitis/chronic obstructive pulmonary disease. Kidney tissues included kidney with chronic lymphocytic interstitial nephritis. Also examined were livers with chronic inflammation and cirrhosis due to chronic hepatitis C infection, autoimmune hepatitis, or alcoholic cirrhosis. In biopsies of lung with chronic bronchopneumonia (Fig. 1, A–F), VE-JAM/JAM 2 was expressed in the endothelium of small-, medium-, and large-caliber arterioles present.

![FIGURE 1. VE-JAM/JAM 2 mRNA expression in inflamed human lung and testicular seminiferous tubules. VE-JAM/JAM 2 was expressed in small- and medium-caliber arterioles in the interstitium within areas of inflammation in the lungs with chronic bronchopneumonia, as shown by the paired bright and dark field images of H&E-counterstained sections (A and B; C and D). Expression was also present in submucosal arterioles in inflamed bronchi (E and F). In contrast, no VE-JAM/JAM 2 mRNA expression was observed in normal lung specimens (G and H). VE-JAM/JAM 2 mRNA expression was also seen on the epithelium in testicular seminiferous tubules (I and J).](http://www.jimmunol.org/Downloadedfrom)
within, or immediately adjacent to, foci of lymphocytic inflammation. This VE-JAM/JAM 2 expression was not observed in normal lungs (Fig. 1, G–H). Kidney with chronic lymphocytic interstitial nephritis and liver with chronic lymphocytic hepatitis were also examined. While VE-JAM/JAM 2 expression was again restricted to the endothelium of arterioles in and adjacent to sites of lymphocytic inflammation, no VE-JAM/JAM 2 expression was found in the liver specimen (data not shown), suggesting VE-JAM/JAM 2 mRNA expression may not be inducible in all inflammatory tissue types.

**Localization of VE-JAM/JAM 2 in primary neoplasms**

The expression of VE-JAM/JAM 2 mRNA was also examined in a number of primary neoplasms (breast carcinoma, pulmonary squamous cell carcinoma, pulmonary adenocarcinoma, prostatic adenocarcinoma, colonic adenocarcinoma). Discrete VE-JAM/JAM 2 expression was seen in the endothelium of small- and medium-caliber arterioles in the following tissue samples: colonic adenocarcinoma (five of six cases, data not shown), testicular carcinomas (two of two cases; Fig. 2, A and B), pulmonary adenocarcinoma (three of five cases; Fig. 2, C and D), and mammary adenocarcinoma (one of three cases; Fig. 2, E and F). The expression of VE-JAM/JAM 2 is highly selective, as illustrated on a low magnification survey or a breast carcinoma (Fig. 2, G and H, *) with adjacent normal breast tissue (Fig. 2, G and H, denoted by an arrow). The expression of VE-JAM/JAM 2 is observed only in vessels adjacent to the tumor site (Fig. 2, G and H, tumor denoted by arrowheads); no VE-JAM/JAM 2 staining could be observed in the normal breast tissue.

**VE-JAM/JAM 2 is not expressed on peripheral blood cells, but interacts with NK, NK/T, and cytolytic T cells**

Having identified VE-JAM/JAM 2 expression on certain isolated endothelial cell types, we hypothesized VE-JAM/JAM 2 as a leukocyte trafficking/adhesion molecule. To examine this, we generated a biotinylated VE-JAM/JAM 2/human IgG fusion protein (VE-JAM/JAM 2.Fc) and isolated the VE-JAM/JAM 2-interacting peripheral blood leukocytes using streptavidin-conjugated magnetic beads. Isolated cells were then examined for surface CD-Ag expression. Comparing results obtained with the biotinylated VE-JAM/JAM 2.Fc with those obtained with biotinylated human IgG, four cell populations stood out. They were CD3+ cells (2.39% for human IgG, 20.99% for VE-JAM/JAM 2), CD8+ cells (1.78% for human IgG, 6.68% for VE-JAM/JAM 2), CD19+ cells (4.42% for human IgG, 9.66% for VE-JAM/JAM 2), and CD56+ cells (6.69% for human IgG, 36.89% for VE-JAM/JAM 2). From these results, we conclude VE-JAM/JAM 2 to interact with peripheral blood T cells (CD3+), B cells (CD19+), and NK cells (CD56+). To confirm VE-JAM/JAM 2 binding to at least one of these cell types, isolated CD56+ NK cells were examined for VE-JAM/JAM 2.Fc interactions by flow cytometry using Alexa-488-conjugated VE-JAM/JAM 2 (VE-JAM/JAM 2.Fc.488). As shown in Fig. 3A, CD56+ NK cells interacted specifically with

**FIGURE 2.** VE-JAM/JAM 2 mRNA expression in primary neoplasia. VE-JAM/JAM 2 mRNA was observed in the endothelium of small- and medium-sized arterioles within tumor samples. Shown are representative paired bright and dark field images of H&E-counterstained sections of testicular carcinoma (A and B), lung adenocarcinoma (C and D), and mammary adenocarcinoma (E and F). Arrows delineate the expression of VE-JAM/JAM 2 mRNA. Example of VE-JAM/JAM 2 mRNA expression on tumor vs normal tissue is shown in low-power magnification (G and H). Breast carcinoma is denoted with an asterisk; normal breast tissue is denoted with an arrow; VE-JAM/JAM 2 expression, as determined by isotopic in situ hybridization, is observed uniquely in vessels adjacent to the tumor (arrowheads), but not in normal tissue.
FIGURE 3. Characterization of peripheral blood VE-JAM/JAM 2 interaction and expression. A, Peripheral blood CD56+ NK cells were obtained by negative selection. NK cells were analyzed by flow cytometry for binding to Alexa-488-conjugated human IgG1 (shaded peak), Alexa-488-conjugated VE-JAM/JAM 2.Fc (solid line), or Alexa-488-conjugated VE-JAM/JAM 2.Fc in competition with unconjugated VE-JAM/JAM 2.Fc (gray line). NK cells readily interact with Alexa-488-conjugated VE-JAM/JAM 2 in solution; this is displaced by the addition of unconjugated VE-JAM/JAM 2 (representative of three independent experiments, 10^5 cells per condition per experiment). B, mAb 12D10.2F9, used at 10 μg/ml, is specific for the VE-JAM/JAM 2-expressing CHO transfectant CHO-JI. The 12D10.2F9 was not observed to interact with the huJAM-expressing CHO transfectant CuL8r. Mouse IgG and the anti-huJAM Ab 10A5 were used at 10 μg/ml. C, PBMC were analyzed for VE-JAM/JAM 2 expression by using mAb 12D10.2F9 at 10 μg/10^6 cells (upper half of the table). In all subsets, no significant VE-JAM/JAM 2 expression was observed. huJAM (shown by mAb 10A5 used at 10 μg/10^6 cells) was found to be expressed on all PBMC subsets (representative of three different experiments). VE-JAM/JAM 2 PBMC interactions were further dissected (lower half of the table) by double staining sorted VE-JAM/JAM 2-binding PBMCs for surface CD-Ag expression. Approximately 30% of total cells were found to bind to VE-JAM/JAM 2.Fc. Of the VE-JAM/JAM 2.Fc-binding PBMC, 50.4% of these were CD56+; of these, 40.2% were CD56+CD8+ and 22.4% were CD56+CD3+. A total of 32.4% of the VE-JAM/JAM 2.Fc-binding PBMC was CD8+; of these, 23.5% were CD8+CD3+ and 73.2% were CD8+CD56+ (representative of three different experiments; “percent positive” represents the percentage of positive cells in an individual staining condition). D, PBDC were obtained from Clonetics and examined for VE-JAM/JAM 2 expression or interaction. DI, VE-JAM/JAM 2.Fc, at 5 μg/10^6 cells, interacted strongly with the PBDC (Alexa-488-conjugated human IgG1, shaded histogram; Alexa-488-conjugated VE-JAM/JAM 2.Fc, solid line; representative of three independent experiments of 10^3 cells per condition per experiment). DII, PBDC were not observed to express VE-JAM/JAM 2 protein (mouse IgG, filled histogram; mAb 12D10.2F9, gray line; representative of three independent experiments of 10^6 cells per condition per experiment).
VE-JAM/JAM 2, the J45 adhesion molecule. A, J45 cells were examined for VE-JAM/JAM 2 binding by flow cytometry. Alexa-488-conjugated VE-JAM/JAM 2.Fc (solid line), used at 0.1 μg/10⁶ cells, shows a significant shift in fluorescence when compared with equivalent Alexa-488-conjugated human IgG1 (filled peak). This shift is blocked by the addition of mAb 12D10.2F9 (gray line) at 20 μg/10⁶ cells. Data are representative of five independent experiments of 10⁵ cells per condition per experiment. B, VE-JAM/JAM 2.Fc or human IgG1, at specified concentrations, were immobilized via the Fc domain onto 96-well plates. BCECF AM-labeled J45 cells demonstrate concentrations, were immobilized via the Fc domain onto 96-well plates. This adhesion was not observed on the human JAM 2-coated wells (B). Samples were run on a 4–20% SDS-PAGE gel and blotted with streptavidin HRP. As can be seen in lane 2, a unique ~40-kDa streptavidin-reactive band was resolved as the VE-JAM/JAM 2-interacting cell surface molecule.

VE-JAM/JAM 2 does not interact with purified B cells, neutrophils, or monocytes, but VE-JAM/JAM 2 interacts with dendritic cells

To address the difference seen between the VE-JAM/JAM 2-interacting cells obtained by MACS purification and FACS sorting, namely that of the CD19⁺ B cells, purified B cells were generated. These B cells were analyzed for VE-JAM/JAM 2 interaction by flow cytometry and were concluded to be negative (11.56% for HI.488 vs 14.86% for VE-JAM/JAM 2.Fc.488, representative of four independent experiments; data not shown). This finding would suggest that the result obtained through MACS purification is sensitive to cell surface FcR and may represent the error margin inherent in the purification system. Furthermore, neutrophils and CD14⁺ monocytes were reexamined and concluded negative for VE-JAM/JAM 2 interaction (neutrophils, 13.1% for HI.488 vs 13.02% for VE-JAM/JAM 2.Fc.488; monocytes, 12.82% for HI.488 vs 10.65% for VE-JAM/JAM 2.Fc.488, representative of six different experiments, data not shown). Also examined were PBDCs from Clonetics (San Diego, CA). These peripheral blood dendritic cells were found to interact with VE-JAM/JAM 2 (Fig. 3DI, VE-JAM/JAM 2.Fc.488 in solid line, HI.488 in shaded histogram). Taken together, VE-JAM/JAM 2-interacting cell types are as follows: CD56⁺ NK cells, CD56⁺CD3⁺ NK/T cells, CD56⁺CD3⁻CD8⁺ cytolytic T cells, and PBDCs. Much like the peripheral cell types examined, PBDCs had no detectable VE-JAM/JAM 2 expression (Fig. 3DI, mouse IgG in filled histogram, 12D10.2F9 in gray line).
attempted to duplicate this by using J45, a CD3⁺ T cell line. J45 cells have no detectable surface expression of VE-JAM/JAM 2 (data not shown). By flow cytometry, J45 cells were shown to bind VE-JAM/JAM 2 in solution (Fig. 4A: HI.488 in filled histogram, VE-JAM/JAM 2.Fc.488 in solid line, VE-JAM/JAM 2.Fc.488 plus mAb 12D10.2F9 in gray line). Using plate-based adhesion assays, J45 cells were found to adhere to VE-JAM/JAM 2 in an Ab-sensitive fashion (Fig. 4B, human IgG, open bar; VE-JAM/JAM 2.Fc, filled bar; VE-JAM/JAM 2.Fc plus mAb 12D10.2F9, shaded bar). This adhesive event is efficiently inhibited by mAb 12D10.2F9. When comparing the results obtained using the adhesion assay vs the flow cytometric assay, we noticed an inconsistent level of 12D10.2F9 inhibition. In the flow cytometric assays, 12D10.2F9 was capable only of a 50% inhibition, even at concentrations as high as 50 μg/ml. In the adhesion assay, however, 12D10.2F9 was very efficient in the inhibition of VE-JAM/JAM 2-mediated J45 adhesion. One possible explanation for this inconsistency is the contribution of the cell membrane or the cytoskeleton. When J45 cells are interacting with VE-JAM/JAM 2 in solution, a certain cytoskeletal/cell membrane cue that is present in the adhesion assay may be lacking. This cytoskeletal/cell membrane cue may lower the threshold needed for VE-JAM/JAM 2-mediated adhesion, making an Ab more efficient at functional inhibition. Thus, in solution and lacking the cytoskeletal/cell membrane cue, the Ab 12D10.2F9 is inefficient at inhibiting the VE-JAM/JAM 2 association with J45 cells; during adhesion and having the cytoskeletal/cell membrane cue, the Ab becomes efficient enough to inhibit the VE-JAM/JAM 2-mediated J45 adhesion.

Using J45 cells, we began to isolate the cell surface receptor for VE-JAM/JAM 2 binding. By first surface biotinylating, then lysing and immunoprecipitating with an Fc-cross-linked VE-JAM/JAM 2.Fc protein A matrix, we were able to obtain a single avidin-reactive band of ~40 kDa (Fig. 4C, lane 2). This band was not seen in conditions immunoprecipitated with an Fc-cross-linked human IgG/protein A matrix (Fig. 4C, lanes 1 and 3), nor in VE-JAM/JAM 2-immunoprecipitating conditions from the non-VE-JAM/JAM 2-binding B cell line Ramos (Fig. 4C, lane 4).

Identification of the VE-JAM/JAM 2 receptor, JAM 3

While attempting to bulk purify the ~37-kDa VE-JAM/JAM 2-immunoprecipitated band, we chanced upon a protein that was identified as a VE-JAM/JAM 2-interacting protein through expression cloning. This protein, found to be a previously cloned molecule termed JAM 3 (accession number NP_113658), is a member

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** VE-JAM/JAM 2 and JAM 3 interactions. A, huJAM 3 sequence is represented. ▲, Putative signal cleavage site; ●, conserved extracellular cysteines. The transmembrane domain is underlined; dotted lines overlie the potential N-glycosylation sites. B, Plate-bound JAM 3.Fc or huJAM.Fc were exposed to biotinylated VE-JAM/JAM 2.Fc (VE-JAM/JAM 2.Fc biotin, used at specific microgram per milliliter concentrations) in the presence of 0.25 μg/well mouse IgG or the anti-JAM 3 Ab MaJIR1. Streptavidin HRP was used to detect; results were obtained from enzymatic color change assay. JAM 3.Fc-coated wells demonstrated VE-JAM/JAM 2.Fc biotin binding (●); huJAM.Fc-coated wells (●) did not. Mouse IgG (●) had no effect, while the anti-JAM 3 Ab MaJIR1 (▲) inhibited VE-JAM/JAM 2 binding. Data are representative of four independent experiments; error bars represent the SD in an n = 4 condition. C, VE-JAM/JAM 2.Fc were captured onto a plate, and biotinylated JAM 3.Fc (JAM 3.Fc biotin) was used, at specified μg/ml concentrations, to examine VE-JAM/JAM 2 and JAM 3 interaction. Mouse IgG or MaJIR1 were used at 0.5 μg/ml. The anti-JAM 3 Ab MaJIR1 was again able to inhibit the VE-JAM/JAM 2 and JAM 3 interaction. Data are representative of three independent experiments; error bars represent the SD in an n = 4 condition.
of the A33/JAM family of Ig superfamily proteins. Like huJAM, A33, and VE-JAM/JAM 2, JAM 3 contains a single predicted transmembrane domain, two putative Ig loops, and a short predicted cytoplasmic C terminus (Fig. 5A). Abs against a 6×His-tagged JAM 3 extracellular domain were raised; one particular clone, in this study termed MaJIR1 (mouse anti-JAM 3), was singled out for its inhibitory activity.

Using MaJIR1 (anti-JAM 3), purified VE-JAM/JAM 2, and JAM 3 fusion proteins, we quickly reproduced the expression cloning results in a plate-based assay format. VE-JAM/JAM 2 was demonstrated to interact with JAM 3. Anti-JAM 3 Ab blocked this interaction. No significant binding of VE-JAM/JAM 2 was detected to huJAM (Fig. 5, B and C). VE-JAM/JAM 2 interactions with JAM 3 in this format were also blocked by the anti-VE-JAM/JAM 2 Ab 12D10.2F9 (data not shown).

JAM 3 expression on J45 cells and CD56⁺ NK cells was confirmed by functional assays and bolstered by immunoprecipitation/Western blotting experiments. The immunoprecipitation/Western blotting experiments confirmed JAM 3 as the ~40-kDa VE-JAM/JAM 2-interacting band that was described in J45 cells above (Fig. 6A, lane 6; lane 5 represents the human IgG/protein A control). PBMC treated in a similar fashion also yielded JAM 3 as the VE-JAM/JAM 2-interacting protein (Fig. 6A, lane 2; lane 1 representing the human IgG/control A control). VE-JAM/JAM 2.Fc protein A matrix showed no cross-reactivity to MaJIR1 in the ~40-kDa region (Fig. 6A, lane 7). JAM 3 was also identified as the adhesion mediator in VE-JAM/JAM 2-dependent J45 adhesion, as anti-JAM 3 was able to inhibit VE-JAM/JAM 2-mediated J45 adhesion (Fig. 6B). JAM 3 was also shown to be the VE-JAM/JAM 2 receptor on CD56⁺ PBLs, as excess free JAM 3 succeeded in inhibiting VE-JAM/JAM 2 binding by CD56⁺ PBLs (Fig. 6C, histograms as labeled). In similar experiments, anti-JAM 3 was also shown to be able to inhibit VE-JAM/JAM 2 binding to CD56⁺ PBLs (data not shown).

**Discussion**
uJAM (F11-Ag), reported previously by various groups (9–13), is a member of an expanding family of adhesion molecules that we termed the A33/JAM family of adhesion molecules. By scanning expressed sequence tag databases for homologies to this A33/JAM family and through expression cloning experiments we have successfully characterized two interacting members of the A33/JAM family of adhesion molecules, VE-JAM/JAM 2 and JAM 3. VE-JAM/JAM 2 was previously described (1, 2), while JAM 3 (accession number NP_113658) has to date been uncharacterized.

In this study, we give a brief description pertaining to VE-JAM/JAM 2 localization by in situ hybridization in both normal and diseased human tissue. In normal fetal tissue, VE-JAM/JAM 2 is present in the vascular endothelium of small and large vessels, while in normal adult tissue, VE-JAM/JAM 2 is found in the endothelium of tonsillar and lymphatic HEV, as previously reported (1, 2). We also identified VE-JAM/JAM 2 mRNA expression the spermatogenic cells of the epithelium in testicular seminiferous tubules, and in the intermediate trophoblasts of placenta for the first time. Also for the first time, we observe VE-JAM/JAM 2

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**FIGURE 6.** JAM 3 is the J45 VE-JAM/JAM 2-binding protein. A, PBMC (lanes 1 and 2), Ramos (lanes 3 and 4), or J45 (lanes 5 and 6) cells were lysed and immunoprecipitated against human IgG (lanes 1, 3, and 5) or VE-JAM/JAM 2.Fc (lanes 2, 4, and 6) Fc-cross-linked protein A matrices. Samples run on a 4–20% SDS-PAGE gel were blotted with 10 μg/ml MaJIR1. A unique ~40-kDa, MaJIR1-reactive band appeared under both the PBMC and J45 VE-JAM/JAM 2.Fc immunoprecipitation conditions; this band is not caused by the VE-JAM/JAM 2.Fc matrix (lane 7). B, Using MaJIR1, we can demonstrate an inhibition of VE-JAM/JAM 2-dependent J45 adhesion. Data are representative of three independent experiments; error bars represent the SD in an n = 6 condition. C, Using 6×His-tagged JAM 3 protein, we can compete off the binding between CD56⁺ NK cells and VE-JAM/JAM. Alexa-488-conjugated VE-JAM/JAM 2.Fc-only condition represented in the lower left histogram; 6×His-tagged JAM 3 containing Alexa-488-conjugated VE-JAM/JAM 2.Fc condition represented in the lower right histogram. Data are representative of three independent experiments.
expression in tumor and chronically inflamed samples. VE-JAM/JAM 2 was found in the endothelium of small-, medium-, and large-caliber arterioles within or around foci of lymphocytic inflammation in some tissues (of the tissues examined, VE-JAM/JAM 2 expression as described was seen in lung and kidney but not in liver). In samples of primary neoplasm, VE-JAM/JAM 2 expression was seen in the endothelium of small- and medium-caliber arterioles within or around tumor foci (breast carcinoma, pulmonary squamous cell carcinoma, pulmonary adenocarcinoma, prostatic adenocarcinoma, colonic adenocarcinoma). These findings confirm VE-JAM/JAM 2 localization (1, 2), furthering the understanding of VE-JAM/JAM 2 localization beyond that which was previously reported, and demonstrate, for the first time, epithelial localization of VE-JAM/JAM 2.

Because VE-JAM/JAM 2 message was observed by in situ hybridization in the aforementioned inflammatory tissues, we had attempted to stimulate VE-JAM/JAM 2 protein expression by cytokine/chemokine treatment of cultured endothelial cells (HUVECs). The following cytokines/chemokines were tested: TNF-α, TGF-β, IL-1β, IL-4, IFN-γ, Exodus-2, stromal cell-derived factor-1α, stromal cell-derived factor-1β, and GM-CSF. No significant up-regulation of surface VE-JAM/JAM 2 protein expression was observed on HUVECs treated for up to 72 h (our unpublished observations). Such results led us to conclude that the cytokines/chemokines used cannot up-regulate VE-JAM/JAM 2 on the surface of HUVECs in a cultured, in vitro setting. However, as the responsiveness of endothelial cells to cytokines/chemokines can be drastically different in an in vitro vs an in vivo setting, the lack of VE-JAM/JAM 2 protein up-regulation in vitro is not unexpected. Such data may point to a more complicated regulatory pathway that needs to take place in vivo before VE-JAM/JAM 2 protein up-regulation can be observed.

Also described for the first time is the interaction of VE-JAM/JAM 2 protein with CD56+ cells. VE-JAM/JAM 2, not detected on any subsets of peripheral blood cells by flow cytometry, was found to interact specifically with CD56+ NK cells, CD56+CD3+ NK T cells, CD56+CD3+CD8+ cytotoxic T cells, and PBDCs. These findings further support the potential role of VE-JAM/JAM 2 as a participant in immune surveillance and Ag-dependent immune responses. These suppositions were further supported by the findings describing VE-JAM/JAM 2 as an adhesion mediator for J45 T cells. By immunoprecipitating with a VE-JAM/JAM 2-Fc protein A matrix, we were able to further identify a single ~40-kDa cell surface molecule on J45 cells as the VE-JAM/JAM 2-interacting protein. This ~40-kDa J45 cell surface molecule was confirmed to be JAM 3 through immunoprecipitation/Western blotting experiments. This led us to hypothesize and prove, through both protein/protein and protein/cell assays, that JAM 3 was the receptor for VE-JAM/JAM 2-dependent adhesion on J45 cells and the VE-JAM/JAM 2-binding receptor on CD56+ PBLS.

Certain observations from the experimental results gave some insight into the interaction between VE-JAM/JAM 2 and JAM 3. Also synthesized were 8×His-tagged VE-JAM/JAM 2 fusion proteins. However, regardless of where the tag was applied, the 8×His-tagged version of the VE-JAM/JAM 2 fusion proteins was more reactive to mAb 12D10.2F9 but did not interact with JAM 3. Similarly constructed JAM 3/8×His-tagged fusion proteins were fully functional, binding to JAM 2-Fc. Also generated were Fab-like fragments of the VE-JAM/JAM 2-Fc. These, again, were reactive to mAb 12D10.2F9 but did not interact with JAM 3. The two observations stated above can suggest that VE-JAM/JAM 2 is required to be at least a dimer for proper function, an observation previously seen with huJAM (9, 14). This hypothesis can also explain why VE-JAM/JAM 2-Fc is required to be cross-linked/captured by a secondary Ab before it can support J45 cells adhesion.

With the findings reported in this work, we believe we have furthered the understanding of VE-JAM/JAM 2 function. In this study, we have identified a candidate receptor/ligand pair that may be involved in the process of the T lymphocyte, NK cell, and dendritic cell trafficking and recruitment to sites of inflammation.

References