Activated leukocyte cell adhesion molecule is a component of the endothelial junction involved in transendothelial monocyte migration

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Abstract Transendothelial leukocyte migration is a major aspect of the innate immune response. It is essential in repair and regeneration of damaged tissues and is regulated by multiple cell adhesion molecules (CAMs) including members of the immunoglobulin (Ig) superfamily. Activated leukocyte cell adhesion molecule (ALCAM/CD166) is an Ig CAM expressed by activated monocytes and endothelial cells. Hitherto, the functional relevance of ALCAM expression by endothelial cells and activated monocytes remained unknown. In this report, we demonstrate soluble recombinant human ALCAM significantly inhibited the rate of transendothelial migration of monocyte cell lines. Direct involvement of ALCAM in transendothelial migration was evident from the robust inhibition of this process by ALCAM blocking antibodies. However, soluble recombinant ALCAM had no impact on monocyte migration or adhesion to endothelium. Localization of ALCAM specifically at cell–cell junctions in endothelial cells supported its role in transendothelial migration. This study is the first to localize ALCAM to endothelial cell junctions and demonstrate a functional relevance for co-expression of ALCAM by activated monocytes and endothelial cells.
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1. Introduction

Migration of monocytes from circulation to extravascular tissues is critical for generating tissue monocytes and macrophages, which are an integral feature of both innate and adaptive immune responses, and the repair process in many tissues. Monocyte recruitment is a complex process that involves multiple adhesive interactions with the endothelium [1–3]. Early stages in this process involve rolling of circulating monocytes in post capillary venules in the systemic circulation. A marginating pool of monocytes physically deform to transit pulmonary capillary vessels and are therefore routinely slowed-down in this vascular segment, which is the primary site for their retention in the lung [4,5].

The initial relatively weak adhesion characteristic of rolling is followed by markedly stronger adhesion between monocytes and endothelial cells mediated by leukocyte-specific β2 integrins and cognate ligands such as intercellular cell adhesion molecule family members expressed on endothelium. Adherent monocytes become activated and upregulate expression of several adhesion molecules, which promotes their migration through endothelial junctions, or through the body of endothelial cells in a process called diapedesis. Several adhesion molecules play important roles in this final stage of recruitment. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD31), is expressed on the surfaces of most leukocytes and at the borders of endothelial cell contacts, and is required for monocyte diapedesis [6]. Interestingly, inhibition of PECAM-1 function with blocking antibodies [7] or by genetic ablation [8] does not effect diapedesis in several vascular beds including the pulmonary microcirculation [7]. CD99 is expressed on subsets of leukocytes as well as at endothelial cell junctions, and has been shown to control the final step of monocyte diapedesis across human umbilical vein endothelial cell barriers [9]. More recently, the role of junctional adhesion molecules (JAM) in diapedesis have been documented [10–15]. There is robust recruitment of neutrophils by inflamed lungs in transgenic mice over-expressing endothelial JAM-C [16], although JAM-C over-expression has no significant impact on monocyte recruitment [16]. These findings are consistent with a model whereby diapedesis is tightly controlled at the local level by several factors including adhesion molecules tethered at endothelial junctions that exert a unique barrier phenotype in different vascular segments [17].

ALCAM (CD166) is an immunoglobulin (Ig) cell adhesion molecule consisting of five extracellular Ig domains, a single transmembrane domain and a short cytoplasmic tail. It is expressed by several cell types including endothelial cells, and given multiple names including BEN and HB2 [18–21]. ALCAM is not expressed in peripheral blood monocytes, however, it is upregulated in activated monocytes [22,23]. The functional relevance of this induction currently remains poorly understood. ALCAM has been implicated in invasion of endothelial cells into cartilage [24], implantation of blastocysts [25], neurite outgrowth [26] and invasion of melanoma cells [27]. While each of these studies implicates ALCAM in cell migration, a similar role for the molecule in activated monocytes has not previously been reported. In this study, we show that soluble recombinant AL-

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CAM (ALCAM-Fc) markedly inhibited transendothelial migration of THP1 and HL60 monocytes, but had no impact on migration or adhesion of monocytes to endothelium. Confocal analysis showed localization of ALCAM at endothelial cell junctions consistent with its specific role in diapedesis. Moreover, ALCAM-GFP was recruited to cell–cell contacts in live endothelial cells. These findings represent the first demonstration of ALCAM's subcellular localization in endothelial cells and its role in diapedesis.

2. Materials and methods

2.1. Human cell lines

Human monocyte cell lines THP1 (catalog no. TIB-202), HL-60 (catalog no. CCL-240) and U937 (catalog no. CRL-1593) were purchased from American Type Culture Collection (ATCC), Manassas, VA. The human K562 erythroleukemia cell line was a gift from Dr. Pace. THP1 cells were cultured in RPMI 1640 medium (ATCC), supplemented with 10% fetal bovine serum (FBS) and 0.05 mM 2-mercaptoethanol. HL60 cells were cultured in RPMI 1640 medium (ATCC) containing 10% FBS. HL60 and K562 cells were cultured in Iscove's modified Dubelcco's medium (IMDM) (ATCC) containing 10% FBS.

2.2. Isolation and culture of primary rat endothelial cells

Pulmonary microvascular endothelial cells (PMVECs) were isolated and cultured using a modification of a method we have previously described [28]. Male Sprague-Dawley rats (300–400 g) were euthanized by intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal, Abbott Laboratories). After sternotomy, the heart and lungs were removed en bloc and placed in a DMEM bath containing 90 µg/ml penicillin and streptomycin. Thin strips were removed from the lung periphery adjacent to the pleural surface, finely minced, and transferred with 2–3 ml DMEM to a 15-ml conical tube containing 3-ml digestion solution. [0.5 g BSA, 10 000 U type 2 collagenase (Worthington Biochemical Co, Lakewood, NJ), and cmf-PBS (Gibco BRL) to make 10 ml total volume]. The digestion mixture was allowed to incubate at 37 °C for 15 min before pouring through an 80-mesh sieve into a sterile 200-ml beaker. An additional 5 ml of normal medium [10% fetal bovine serum (FBS), Hyclone, Logan, UT) with 30 µg/ml penicillin and streptromycin in DMEM] was used to wash the sieve. The isolation mixture was transferred to a 15 ml conical tube and centrifuged at $300 \times g$ for 5 min, the medium aspirated, and the cells resuspended with 5 ml complete medium [1 part microvascular conditioned medium: three parts incomplete medium (80% RPMI 1640, 20% FBS, 12.3 U/ml Heparin (Elkins-Sinn, Cherry Hill, NJ), and 6.7 µg/ml Endogro (Vec Technologies, Rensselaer, NY) with 30 µg/ml penicillin and streptromycin]. Centrifugation/aspiration was repeated, the cells resuspended in 2–3 ml complete medium and allowed to incubate at 37 °C for 30 min before being placed drop wise onto 35-mm culture dishes. After 1 h at 37 °C with 5% CO$_2$, 3 ml of complete medium was added. The dishes were checked daily for contaminating cells that were removed by scraping and aspiration. Endothelial cell colonies were isolated with cloning rings, trypsinized, resuspended in 100 µl complete medium and placed as a drop in the center of a T-25 flask. The cells were allowed to attach (1 h at 37 °C with 5% CO$_2$) before the addition of 5 ml complete medium. Cultures were characterized using, uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled low-density lipoprotein (DiI-acetylated LDL), a lectin-binding panel and were routinely passaged by scraping [29].

2.3. Chimeric molecules and antibodies

Recombinant human ALCAM-Fc, recombinant mouse ALCAM-Fc and recombinant human VE-cadherin-Fc chimeric proteins were purchased from R&D Systems (Minneapolis, MN). Generation of rabbit anti-rat ALCAM antibody BRI-1 has previously been described as anti-HB2 [21,30]. BRI-1 antiserum was purified on a protein-A column (ImmunoPure Plus High Capacity, Pierce Biotechnology, Inc. Rockford, IL). Monoclonal anti-ALCAM antibodies used were anti-human clone MOG/07 (Novacastra, Newcastle, UK), anti-rat ALCAM clone 2117 (gift from Genetech) and purified and FITC-labeled anti-human ALCAM clone J4-81 (Antigenix America, Huntington Station, NY).

Horse radish peroxidase (HRP) conjugated antibodies used included anti-mouse and -rabbit IgG (Santa Cruz Biotech and Jackson Laboratories, West Grove, PA). Alexafluor 488 and 594 were from Molecular Probes, (Eugene, OR). Fab fragments were made by incubating monoclonal antibodies with immobilized Papain beads (Pierce, Biotechnology, Rockford) for 3 h at 37 °C, according to the manufacturer's protocol; uncut IgG and Fc fragments were then removed by repeated passage over Protein G Sepharose. Purity and proper size of IgG and Fab fragments were confirmed by SDS–PAGE. Anti-human IgG (Fc-specific) Fab was purchased from Sigma (St Louis, MO) and FITC-labeled mouse IgG1 from Antigenix America.

2.4. Flow cytometry

THP1 and HL60 monocytes were washed once in IMDM with 5% FBS, then resuspended in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (FACS buffer) with saturating amounts of human Fc receptor block (Miltenyi Biotech Inc. Auburn, CA). After five-minute incubation on ice, FITC-labeled anti-human ALCAM antibody clone J4-81 or an isotype-matched control (FITC-labeled mouse IgG1) was added to the cells for 30 min. After 30 min of incubation on ice, cells were washed three times and then resuspended in FACS buffer and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). Additional control cell suspensions were incubated with secondary antibody alone.

2.5. Cell fractionation and Immunoblotting

Cell lysates prepared with ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 1% triton X-100 (v/v) and supplemented with 1% protease inhibitor cocktail (Roche, Indianapolis, IN) was clarified by centrifugation at 13 000 rpm for 15 min at 4 °C, and soluble cell fractions harvested. Protein content in cell lysates was measured using a Lowry protein assay (Sigma). Lysates were combined with Laemmli buffer (Sigma), boiled for 2 min and resolved by electrophoresis on a 10% polyacrylamide gel. Samples were blotted unto nitrocellulose membranes, probed with antibodies, and protein bands identified by chemiluminescence (Fujifilm LAS-1000 imaging system, FujiFilm, Valhalla, NY).

2.6. Immunostaining

Endothelial cells seeded on glass cover slips were fixed with methanol and blocked with 2% normal goat serum for 10 min, followed by staining with affinity purified BRI-1 (1: 200 dilution), and Alexflour 594 (1/1000 dilution) (Molecular Probes). Cells were mounted with Dako Fluorescent mounting media (Dako, Carpinteria, CA). For negative control, the steps with primary antibody were omitted, and no specific immunoreactivity was detected in those slides. Stained cells were visualized using a laser confocal scanning microscope (Leica TCS SP2, Leica, Exton, PA) and by epifluorescence (Nikon TE2000, Nikon Instruments Inc., Melville, NY).

2.7. Cloning and expression of ALCAM-GFP

ALCAM cDNA was amplified from total RNA isolated from cultured rat PMVECs using gene-specific primers flanked with recognition and cleavage sites for Age I and SaI I. (Forward primer; 5′-TTGTCGGTGGCCTTCTAGGA-3′, reverse primer; 5′-GGCTTCTGTTTTGTGATTG-3′). PCR product was sub-cloned into the *Age*I/*Sal*I site in pRV-CMV-eGFP vector (Applied Vironomics, Fremount, CA). Multiple clones were isolated and verified by sequence analysis. For transfection, log-phase growing human K562 cells and semi-confluent rat PMVECs seeded on glass cover slips were transfected with ALCAM-GFP plasmid DNA (2.5 µg) using lipofectamine 2000 (Invitrogen). Stable lines of K562-ALCAM-GFP and ALCAM-GFP were selected with G418 (700 µg/ml) for 20 days. Expression of ALCAM-GFP was examined by epifluorescence (Nikon TE2000). MetaMorph premier software was used to obtain z-stacks of K562-ALCAM-GFP clusters.

2.8. Monocyte–endothelial cell adhesion

PMVECs were grown to confluence in 96-well plates. THP1 monocytes were labeled with 5 µg/ml calcein-AM (Molecular Probes) at 37 °C for 20 min, and washed twice with phenol-red free IMDM containing 10% FBS. Fluorescent-labeled THP1 monocytes (10^4/well) were added to PMVEC monolayers at 37 °C for 30 min in the presence

or absence of recombinant human ALCAM-Fc (25 μg/ml). After washing, adhesion of monocytes was detected by excitation at 490 nm with bandwidth of 5 nm, and emission at 510 nm with a bandwidth of 5 nm at 37 °C using a bottom reading fluorescent plate reader (Safire[2] Tecan, Salzburg, Austria).

2.9. ALCAM ligand binding

Flat bottom 96-well plates were coated with goat anti-human Fc-F(ab′)$_2$ in binding buffer (20 mM Tris, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, pH 8.0) for 1 h. Thereafter, the wells were treated with 1% (w/v) bovine serum albumin in binding buffer and coated with 500 ng/ml of recombinant human ALCAM-Fc for 1 h. K562 cells expressing rat ALCAM-GFP or GFP alone (2×10^4) were allowed to adhere to recombinant human ALCAM-Fc coated plates for 30 min at 37 °C. After washing, adhesion of K562 cells was detected by excitation at 490 nm with bandwidth of 5 nm, and emission at 510 nm with a bandwidth of 5 nm at 37 °C using a bottom reading fluorescent plate reader (Safire[2] Tecan).

2.10. Migration

Fluorescently labeled THP1 monocytes (10^4) in phenol-red free and serum-free IMDM (200 μl), in the presence or absence of recombinant human ALCAM-Fc (25 μg/ml), were added to the upper chamber in Floroblock transwells inserts with 3 μm pore size. Phenol-red free and serum-free IMDM (700 μl) with 10 ng/ml of MCP-1 was placed in the bottom chamber. Migration of THP1 cells to the bottom chamber was detected by excitation at 490 nm with bandwidth of 5 nm, and emission at 510 nm with a bandwidth of 5 nm at 37 °C using a bottom reading fluorescent plate reader (Safire[2] Tecan).

2.11. Transendothelial migration

Endothelial cells (50 000/well) were seeded on 3 μm pore-size fluorescent blocking membrane inserts (BD Bioscience, Bedford, MA) without media in the bottom chamber to prevent migration through the pores. Culture media was changed every three days, and confluent endothelial monolayers gently rinsed with phenol-red free IMDM containing 10% FBS. The endothelial monolayer was loaded with fluorescently labeled monocyte cell lines (10^6) then transferred to fresh Transwell containing phenol-red-free IMDM with MCP-1 (0–100 ng/ml). Transwells were incubated at 37 °C in a humidified incubator with 5% CO$_2$, and migration of monocytes through the endothelium detected as described above using a bottom reading fluorescent plate reader (Safire[2] Tecan).

2.12. Statistics

The data are reported as the means ± S.D. for at least three independent experiments. Statistical analysis of the raw data were performed by two tailed t tests. Student's t-test was used to measure differences in samples of two groups. Probability of less than 0.05 was considered significant.

3. Results and discussion

ALCAM is expressed by activated monocytes and endothelial cells however the functional relevance of this co-expression has not yet been explored. In this work we demonstrated that ALCAM is required for transmigration of monocyte cell lines, however, ALCAM was not involved in adhesion of monocytes to endothelium. These functional data were supported by specific localization of ALCAM at cell–cell junctions in endothelial cells.

3.1. ALCAM expression in human monocyte cell lines

We initially evaluated the capacity of several antibodies to bind extracellular epitopes of ALCAM by analyzing lysates of Chinese hamster ovary (CHO) cells transfected with plasmid DNA clone Ag 2117, which encodes extracellular rat ALCAM
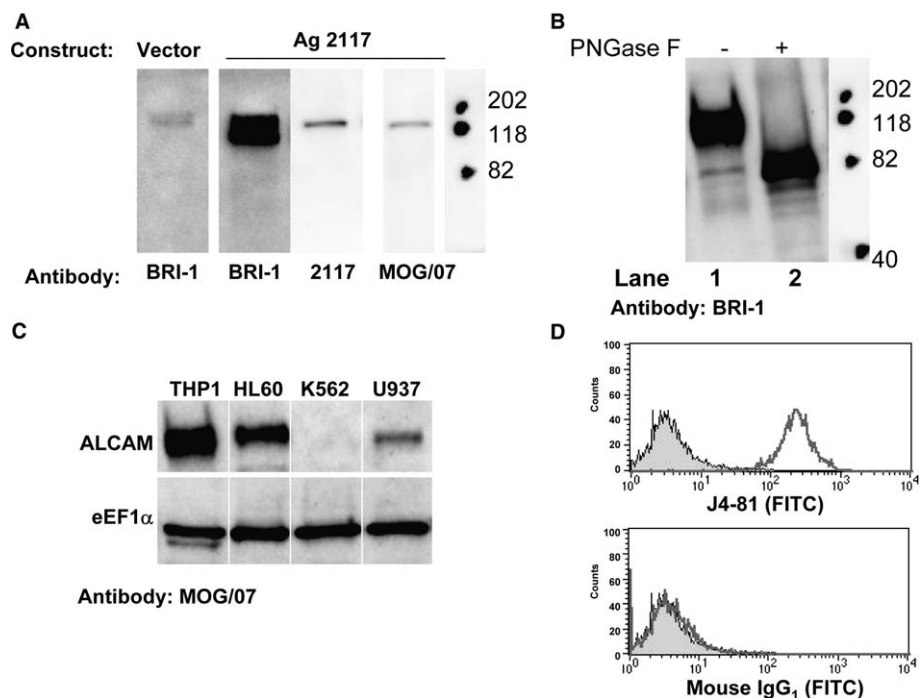

Fig. 1. ALCAM expression by human monocyte cell lines. (A) Lysates of CHO cells transfected with empty vector (vector) or vector expressing extracellular rat ALCAM (Ag 2117) were blotted with anti-ALCAM antibodies BRI-1, 2117 and MOG/07. (B) Treatment of CHO transfectant lysates with PNGase F reduced the size of mature ALCAM (lane 1) to the expected size of the nascent molecule (lane 2). (C) Western blot analysis of ALCAM in human monocyte (THP1, HL60 and U937) and erythroleukemia (K562) cells. Blotting of eEF1α was used to confirm equal loading of protein samples. (D) Flow cytometry identification of ALCAM in THP1 monocytes. Viable cells were gated based on forward and side scatter. Histogram in the upper panel are THP1 monocytes labeled with FITC-conjugated mouse anti-human ALCAM (J4-81), and histogram in the lower panel are cells labeled with isotype-matched mouse IgG$_1$ conjugated with FITC. MW, molecular weight.

as described in a previous study [31]. Immunoblot analysis revealed the presence of a major band of 110 kDa, consistent with the size of mature ALCAM (Fig. 1A). Parental CHO cells contained very low level of endogenous ALCAM as has previously been reported [21]. Fig. 1A shows clearly that this low level expression had no impact on specific detection of rat ALCAM in the CHO transfectants using BRI-1. There was poor reactivity with the monoclonal antibodies, J481 and MOG/07 that were raised against human ALCAM (Fig. 1A and data not shown).

BR-1 reacted strongest and recognized a broad protein band, which on lighter exposure of the immunoblot membrane revealed two distinct bands (data not shown). Nascent ALCAM is 70 kDa in size, and is glycosylated to yield a 90–110 kDa mature molecule, therefore, we considered the possibility that BRI-1 reacted with various ALCAM species. Transfected CHO lysates were digested with N-glycosidase PNGase F (peptide-N-glycosidase F), and the digest blotted with BR-1. A single major protein band was identified in the PNGase F digest (Fig. 1B, lane 2), confirming the specificity of our antibody, and previous studies indicating modification of ALCAM involves addition of N-linked glycan units [20]. Blotting of PNGase digested lysates from parental CHO cells yielded a very faint band of approximately 70 kDa (data not shown), consistent with the relatively low-level ALCAM expression in this cell line (Fig. 1A).

Several myeloid cell lines have been extensively demonstrated to have monocytic characteristics and are routinely used as surrogates for activated monocytes. A previous study of ALCAM mRNA expression revealed ALCAM transcripts in THP1 and HL60 monocyte lines, but not in the U937 monocyte or K562 erythroleukemia cell lines [23]. ALCAM protein expression has hitherto not been demonstrated in these cells therefore, lysates were blotted for ALCAM with anti-human ALCAM monoclonal antibody MOG/07. A major band of about 110 kDa was identified in THP1 and HL60 cells, a faint band of the same size was surprisingly found in U937 cells however there was no protein band in K562 cells (Fig. 1C). Equal loading of protein samples for each cell type was confirmed by the comparable intensities of the house keeping eukaryotic elongation factor 1α (eEF1α) band. Higher level of ALCAM in THP1 monocytes compared to HL60 monocytes, as well as the relatively low-level ALCAM expression in U937 cells were confirmed in multiple experiments. Treatment of cell lysates with PNGase F reduced the size of ALCAM in each monocyte cell line to an immunoreactive protein band of ∼70 kDa (data not shown). Next, we demonstrated by flow cytometry using FITC labeled anti-human ALCAM antibody J4-81, and MOG/07 that ALCAM was expressed on the surface of THP1 and HL60 cells (Fig. 1D and data not shown). These data demonstrated more widespread expression, and appropriate post-translational regulation of ALCAM in human monocyte cell lines, and therefore, these cells were used to evaluate ALCAM's role in monocyte–endothelial interactions.

3.2. ALCAM is involved in transmigration of monocytes across PMVECs

Leukocyte retention and recruitment occurs in the pulmonary capillary bed [4,5]. We and others have extensively studied PMVECs isolated from this vascular segment [28,29,32,33] however there is a paucity of data on monocyte transmigration

across this endothelium. Single cell suspension of PMVECs was seeded on fluorescent blocking transwell inserts and cultured for 4–5 days, creating an endothelial monolayer that was virtually impermeable to 70 kDa dextran solutes (less than 5% permeability, data not shown). THP1 cells were labeled with calcein-AM and added to the upper chamber of the insert. Fluorescence intensity at the bottom of the insert, representing the number of cells that had crossed the endothelial monolayer, was measured at multiple time intervals at 37 °C for 3 h. There was a linear relationship between fluorescence intensity and number of THP1 cells ($R^2 = 0.997$) (Fig. 2A). Moreover, the presence of monocyte chemoattractant protein-1 (MCP-1) in the lower chamber dose-dependently increased the number of THP1 cells that transmigrated across the PMVEC barrier (Fig. 2B). These results demonstrated that our assay supported transendothelial monocyte migration and was therefore suitable for studying the role of ALCAM in this process.

Fig. 2C shows typical profiles of THP1 cell transendothelial migrations performed in the presence or absence of soluble recombinant human ALCAM (ALCAM-Fc). Several studies have previously shown that soluble ALCAM-Fc inhibits ALCAM-mediated cell adhesions [18,24,34], and the preparation used in this study was confirmed by mass spectrometry to be highly pure (data not shown). Transmigration was reduced by 50% in experiments performed in the presence of recombinant human ALCAM-Fc (25 µg/ml) (Fig. 2D). Importantly, recombinant human ALCAM-Fc inhibited transendothelial migration in experiments performed with HL60 cells, indicating the phenomenon we observed with THP1 was not a peculiarity of that monocyte cell line (Fig. 2D). The inhibitory effect of recombinant soluble ALCAM was evident early and persisted throughout the duration of the assay (Fig. 2C). The assay used in this study permitted real-time evaluation of the number of cells migrating through the endothelial barrier therefore we were able to obtain kinetic information on the same population of monocytes traversing the same endothelial monolayer. This data consistently showed fluctuations in the rate of transmigration (Fig. 2C). Importantly, recombinant human ALCAM-Fc did not change the shape of the transmigration curve suggesting the inhibitory effect we observed reflected downregulation of a normal mechanism of monocyte transendothelial migration. In control experiments with recombinant human chimeric Fc protein (VE-cadherin-Fc, 25 µg/ml) no effect on transmigration was identified. This indicates ALCAM-binding molecules on the monocyte cell lines used in this study are involved in transendothelial migration.

To test whether ALCAM was directly responsible for the above transendothelial migrations, various anti-ALCAM antibodies were used to block ALCAM prior to the transmigration assay. Anti-human ALCAM monoclonal antibody J4-81 has previously been demonstrated to block ALCAM mediated functions including proliferation and adhesion [23,35]. When THP1 cells were treated with this antibody, it blocked transmigration by 70%, when compared to experiments performed without any antibody (Fig. 2E). Importantly, J4-81 Fab fragments inhibited transmigration by a similar margin indicating this was a specific function blocking phenomenon, and not a non-specific effect due to potential cross-linking of Fc tails (Fig. 2E). MOG/07 and BRI-1 had relatively modest effects, whereas anti-rat ALCAM monoclonal 2117 and mouse IgG had no significant impact on transmigration (Fig. 2E). Expres-
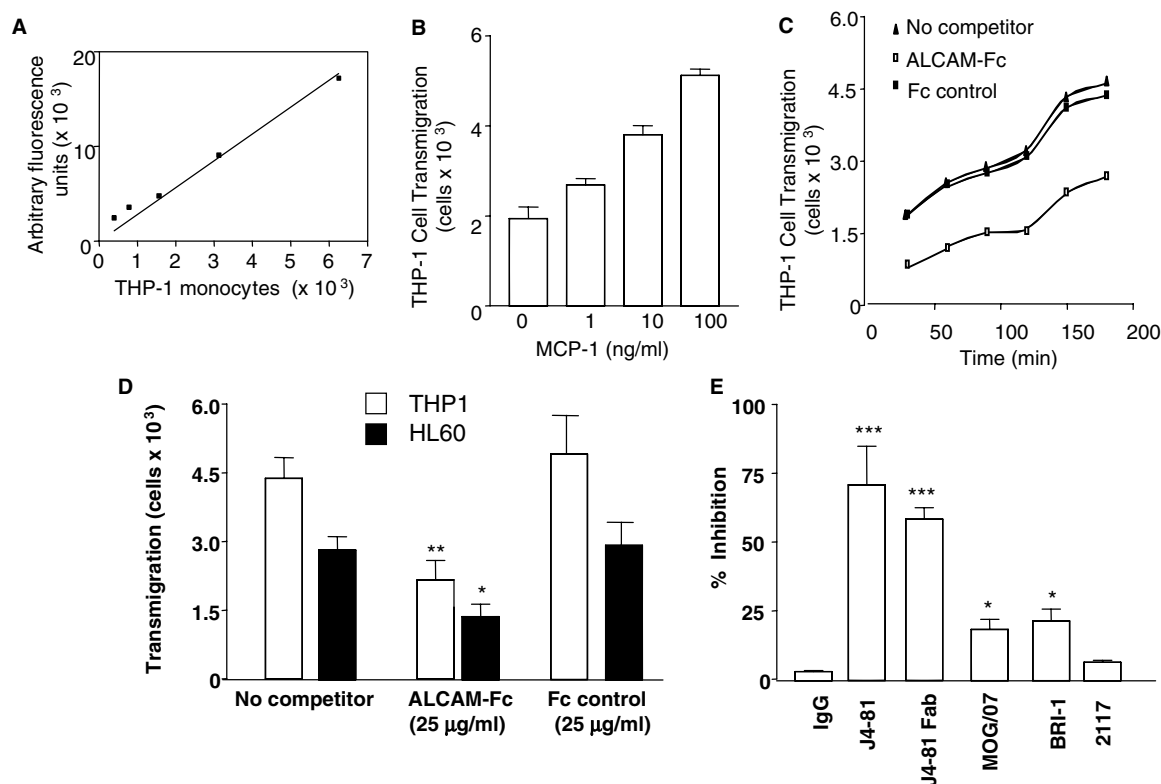
Fig. 2. ALCAM is involved in transendothelial monocyte migration. Fluroblock inserts with 3 μm pores were seeded with PMVECs and confluent endothelial monolayers used for transmigration assays. (A) Standard curve showing linear relationship between fluorescence intensity and number of calcein-AM labeled THP1 monocytes. (B) MCP-1 dose-dependently increased the number of THP1 monocytes that migrated across the endothelial barrier to the bottom of the transwell inserts. (C) Typical transmigration profile of THP1 monocytes in experiments performed in the absence of competitor (control and filled triangle), recombinant human ALCAM-Fc (open box) and recombinant human chimeric Fc-control (filled box). MCP-1 (10 ng/ml) was used as chemoattractant. (D) Quantitative data from experiments similar to that shown in Fig. 3C, showing inhibition of transendothelial migration by ALCAM-Fc (25 μg/ml) and lack of effect in control experiments (No competitor, and Fc-control). Data shown is the mean number of THP1 and HL60 monocytes that transmigrated for 3 h in three independent experiments. $^{**}P < 0.001$, $^{*}P < 0.05$. (E) THP1 monocytes were incubated with 20 μg of J4-81, J4-81 Fab fragments, MOG/07, 2117 and mouse IgG or BRI-1 polyclonal antibody (1:100 dilution) for 30 min. Unbound antibody was washed away, and cells used for transmigration. Blocking ALCAM with J4-81 Fab fragments blocked transmigration as efficiently as when the antibody was used intact. Data shown are the mean number of THP1 monocytes that transmigrated for 3 h in three independent experiments of three replicates. $^{***}P < 0.0001$, $^{*}P < 0.05$.

sion of CD6, the only other known ALCAM ligand is limited to thymocytes, mature T cells, and a small subset of B cells known as B-1 cells [36], and more importantly monocytes lack CD6 expression [37]. Therefore, it is reasonable to conclude that homotypic ALCAM adhesion is primarily responsible for the ALCAM-mediated transendothelial migration reported in this study. ALCAM expression is well characterized in activated primary monocytes and monocyte cell lines, as well as in a small subset of B-cells [21,23]. More recent studies have reported ALCAM expression in circulating dendritic cells [35], and several groups have identified ALCAM as a surface marker of mesenchymal stem cells [24,38–40]. Therefore, ALCAM may be involved in regulating transmigration of diverse cell types across various biological barriers.

3.3. Binding of rat and human ALCAM

The above transendothelial migration data suggested there was functional binding between human and rat ALCAM. Sequence alignment revealed a 95.8% amino acid identity in the N-terminal Ig ligand binding domain (D1), which mediates homotypic ALCAM adhesion (Fig. 3A). This strongly suggested there was conservation of the ligand binding properties

of human and rat ALCAM. Since recombinant human AL-CAM-Fc was used throughout this study, we created recombinant rat ALCAM fused to green fluorescent protein (GFP) to examine binding between the two ALCAM species. We have extensive experience with the human K562 cell line [41–43], which according to several previous studies lacks endogenous ALCAM expression [23,44]. Indeed, ALCAM was not detected in K562 cells by Western blot analysis in this study (Fig. 1C). Parental K562 cells cluster during log-phase growth, nonetheless it was evident more clusters of larger sizes formed in stable K562 cells expressing rat ALCAM-GFP, and moreover ALCAM-GFP was recruited to sites of cell–cell contact (Fig. 3B and data not shown). To test whether rat ALCAM binds human ALCAM, recombinant human ALCAM-Fc was used to coat the bottom of 96-well plates. Single cell suspensions of K562-ALCAM-GFP and control K562-GFP cells were loaded unto recombinant human ALCAM-Fc or BSA coated surfaces, and the number of cells remaining adherent determined by fluorescence intensity. Nearly 18% of stable K562-ALCAM-GFP cells consistently remained attached to recombinant human ALCAM-Fc, compared to the background binding (less than 2.5%) observed for parental K562
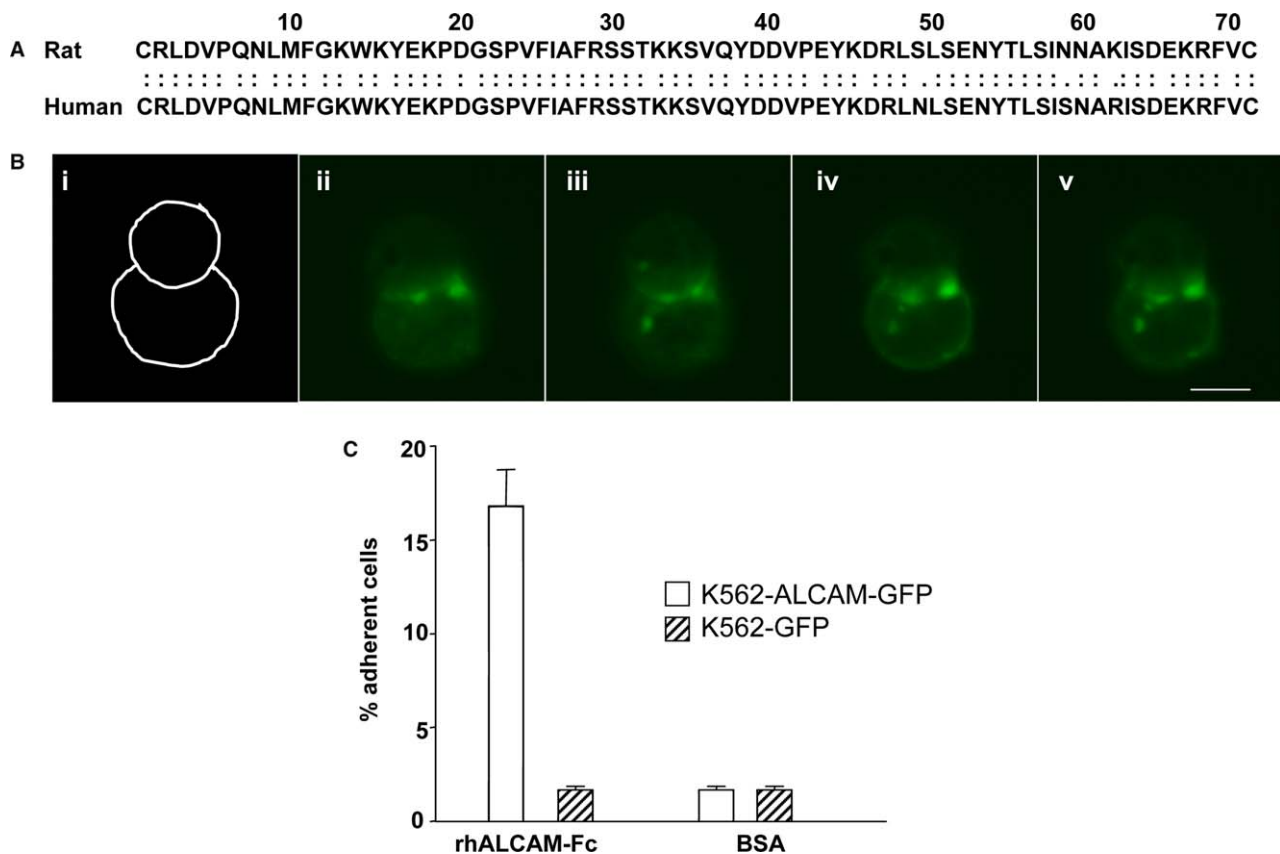
Fig. 3. Binding between rat and human ALCAM. (A) Alignment of the ligand binding N-terminal Ig domain of rat and human ALCAM shows greater than 95% amino acid sequence identity. (B) Cluster of two human K562 cells expressing rat-ALCAM fused to GFP. (i) Outline of the two cells and four consecutive z-sections (ii)–(v) showing localization of ALCAM-GFP in the plasma membrane with enrichment at sites of cell–cell contact. The outline shown in (i) is for cells in the z-section shown in (iii). Bar 25 μm. (C) Stable K562 cells were labeled with calcein-AM and allowed to adhere to immobilized recombinant human ALCAM-Fc (rhALCAM-Fc) at 37 °C for 1 h. Percentage of adhering cells was determined by fluorescence intensity detected by excitation at 490 nm with bandwidth of 5 nm, and emission at 510 nm with a bandwidth of 5 nm a using a bottom reading fluorescent plate reader. Data shown are the mean percentage of cells remaining adherent to immobilized ALCAM ligand in three independent experiments each of eight replicates.

and stable K562-GFP cells (Fig. 3C and data not shown). Similar results were obtained when recombinant mouse ALCAM-Fc was used as the immobilized ligand (data not shown). These data provide direct evidence of ligand binding between human ALCAM and its rat orthologue, and supports the contention that homotypic ALCAM adhesion contributed to the transendothelial monocyte migration reported in Fig. 2.

3.4. ALCAM is not involved in THP1 migration or adhesion to endothelium

It was unclear from the data presented in Figs. 2 and 3 whether ALCAM was involved in diapedesis or firm adhesion of monocytes to endothelium. To investigate the latter more specifically, THP1 cells were labeled with calcein-AM and co-incubated with confluent PMVEC monolayers, seeded on non porous surfaces in 96-well culture plates, in the presence or absence of recombinant human ALCAM-Fc (25 μg/ml). Thereafter, the co-culture was gently rinsed with PBS, and the number of cells adherent to the endothelial monolayer measured by fluorescence intensity. Typically, 40% of THP1 cells remained attached to the endothelium (Fig. 4A). Multiple adhesion assays showed that recombinant human ALCAM-Fc did not alter the percentage of THP1 cells that remained attached to the PMVEC monolayer (Fig. 4A). To verify that

our assay was capable of detecting alterations in THP1 adhesion to endothelium, PMVEC monolayers were pre-treated with tumor necrosis factor α (TNF-α) for 24 h prior to the adhesion assay. Percentage of THP1 cells adherent to TNF-α-treated monolayers increased by 50%, however, ALCAM-Fc had no impact on adhesion (data not shown). Next, we determined whether ALCAM was involved in migration of THP1 monocytes in the absence of an endothelial barrier using the Transwell approach. Significantly more (∼45% more cells) THP1 cells migrated towards MCP-1 in the absence of an endothelial barrier (data not shown). However, there was no statistical difference in the number of cells that migrated in experiments performed in the presence or absence of recombinant human ALCAM-Fc (Fig. 4B and C).

ALCAM has been implicated in motility of endothelial cells and melanoma cells by several groups using the same commercially available recombinant ALCAM-Fc employed in this study to block cell-mediated adhesion [18,24,26,27]. More recently, a truncated soluble ALCAM variant consisting of the N-terminal ligand binding domain alone, reportedly enhanced migration of individual endothelial cells although this reagent had no impact on endothelial sheet migration [45]. It was therefore surprising to find no role for ALCAM in migration of THP1 cells in this study. Recent data suggests that mature den-
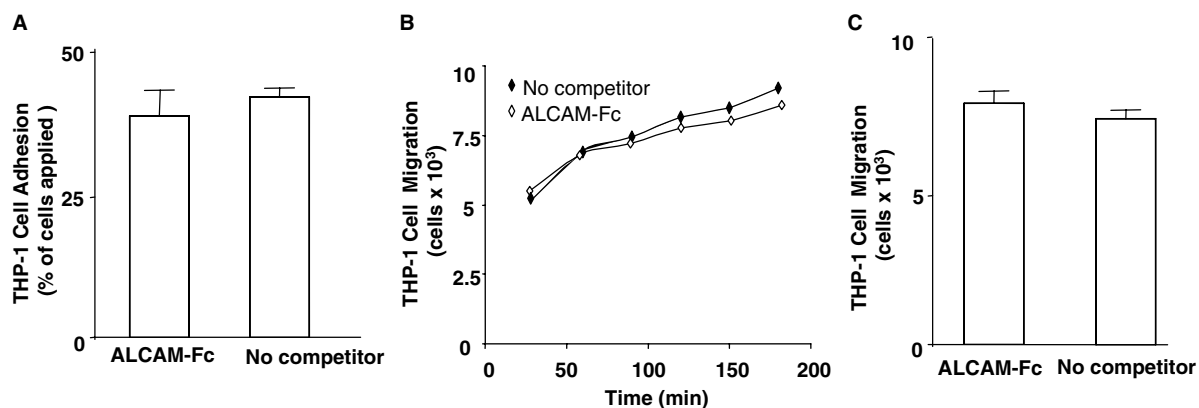
Fig. 4. ALCAM is not involved in monocyte–endothelial adhesion or monocyte migration. (A) Adhesion of calcein-AM labeled THP1 monocytes to PMVEC monolayer in the presence or absence of recombinant human ALCAM-Fc (25 μg/ml). Data shown are the mean percentage of cells remaining adherent to the endothelium in three independent experiments each of at least eight replicates. (B) Typical migration profile of THP1 cells moving through Fluroblock inserts with 3 μm pores towards MCP-1 (10 ng/ml) in the lower chamber. (C) Quantitative data showing THP1 cell migration in the presence and absence of recombinant human ALCAM-Fc (25 μg/ml). Data shown are the mean number of THP1 cells that migrated for three hours in four independent experiments each of three replicates.

dritic cells express an ALCAM variant with a unique surface organization [35], therefore we cannot exclude the possibility that a hitherto unknown modification of ALCAM plays a role in migration of macrophages and dendritic cells, which are more differentiated than the monocytic cells used in this study.

3.5. ALCAM is located in the lateral plasma membrane domain in endothelial cells

Functional data in Figs. 2 and 4 suggested ALCAM was localized at sites in the endothelium specifically involved in diapedesis. We demonstrated by western blot analysis the pres-

ence of fully mature ALCAM in cultured PMVECs (Fig. 5A). Fig. 5B is a panel of confocal lateral photosegments of cultured PMVECs labeled for ALCAM. It shows enrichment of ALCAM in basolateral membrane segments, and sparse labeling in apical and basal domains of the plasma membrane. This sub-cellular localization was confirmed in independent experiments in which both xy and zy-planes of confluent PMVEC monolayers were analyzed by confocal microscopy, and the data showed ALCAM localization in the lateral plasma membrane domain in endothelium (Fig. 5C), which is consistent with a functional role for ALCAM in diapedesis. Treatment
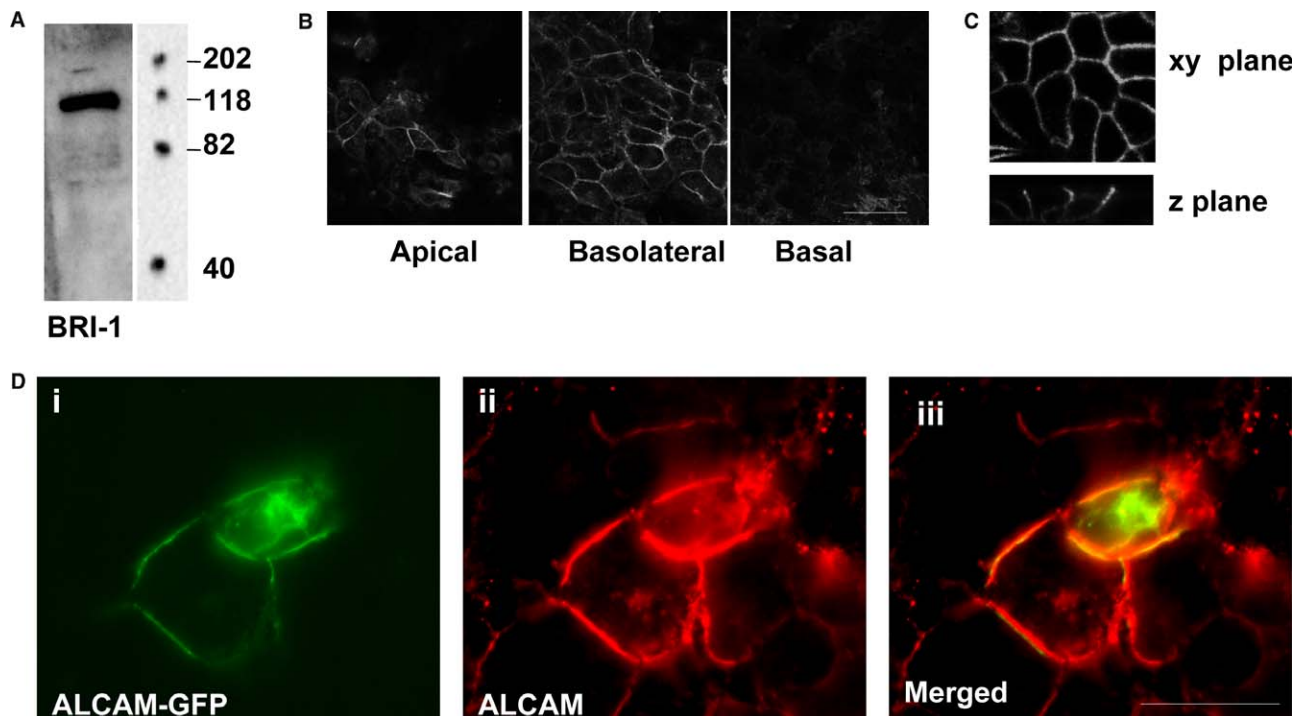

Fig. 5. ALCAM is located at endothelial cell junctions. (A). Western blot analysis showing expression of mature ALCAM in PMVECs. (B) Confocal microscopic photo sections of PMVECs labeled for ALCAM. Images shown here represent apical, basolateral and basal segments taken at 0.3 μm focal intervals (3 of 16 shown here). (C). Confocal z-plane of confluent PMVECs showing ALCAM labeling in lateral plasma membrane domain. (D) ALCAM-GFP (green) localizes to sites of cell–cell contact in transfected PMVECs (i), and co-localizes with endogenous ALCAM (ii) at endothelial cell junctions as shown in the merged image (iii). Bar 50 μm.

of confluent PMVECs with a concentration range (0.5–5 mM) of EGTA delocalized ALCAM from cell junctions, and a putative interaction between ALCAM and the actin cytoskeleton was confirmed by delocalization of the molecule from the endothelial junction following treatment of confluent PMVECs with cytochalasin D (data not shown).

The above data in Fig. 5B and C and experiments with cytochalasin D and EGTA (data not shown) were consistent with recruitment of ALCAM to endothelial cell junctions. Indeed, when PMVECs were transiently transfected with rat ALCAM-GFP vector, ALCAM-GFP was recruited to intercellular junctions in confluent endothelial cultures (Fig. 5Di). Authenticity of the ALCAM molecule expressed by this chimeric construct was confirmed by staining endothelial transfectants with affinity purified BRI-1. Fig. 5Dii shows there is more intense staining of ALCAM in cells expressing ALCAM-GFP compared to cells exclusively expressing endogenous ALCAM. Importantly, ALCAM-GFP behaved exactly like the endogenous protein, and was recruited to endothelial cell junctions.

The central finding in this study is that ALCAM is recruited to the lateral plasma membrane domain in endothelial cells, and at least partially controls diapedesis of ALCAM-positive cells such as the monocyte cell lines used in this study. ALCAM-Fc (25 µg/ml) reduced transmigration by 50%, and while increasing the concentration of ALCAM-Fc to 50 µg/ml did not provide additional inhibition, a lower concentration (5 µg/ml) had an intermediate inhibitory effect blocking transmigration by 33% (data not shown). Direct evidence for ALCAM's role in transendothelial migration was provided by experiments using anti-ALCAM antibody, which revealed inhibition of this process by up to 70%. Since monocytes do not express CD6 [37], the only other known ALCAM ligand, it is reasonable to conclude from these data that homotypic ALCAM-adhesion is responsible for ALCAM-mediated diapedesis. Recent cloning of soluble ALCAM suggests the functional attributes reported for ALCAM and its soluble variant in this study may be physiologically relevant. Interestingly, TNF-α increases soluble ALCAM expression without altering membrane bound ALCAM expression in endothelial cells [45]. Since this cytokine is produced early in the inflammatory response, future studies will test the hypothesis that soluble ALCAM inhibits ALCAM-mediated monocyte recruitment early in the inflammatory response to specifically prevent premature accumulation of tissue macrophages.

The sub-cellular localization of ALCAM in endothelial cells was readily disrupted by subtly disturbing the actin cytoskeleton, and produced a phenotype similar to that reported previously in K562 cells expressing heterologous ALCAM [44,46]. The scaffolding molecule responsible for the interaction between ALCAM and the cytoskeleton remains enigmatic. Recruitment of ALCAM-GFP to endothelial cell junctions in live cells offers a powerful in vivo approach to validate physical coupling between ALCAM and candidate scaffold proteins in future studies. In conclusion, this study provides the first evidence to support a novel concept that ALCAM is a component of the endothelial cell junction and a member of the molecular machinery controlling diapedesis.

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