JAM-L–mediated leukocyte adhesion to endothelial cells is regulated in cis by α4β1 integrin activation

Anny-Claude Luissint,[1,2] Pierre G. Lutz,[3,4] David A. Calderwood,[5] Pierre-Olivier Couraud,[1,2] and Sandrine Bourdoulous[1,2]

[1]Institut Cochin, Université Paris Descartes, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8104, Paris F-75014, France
[2]Institut National de la Santé et de la Recherche Médicale, Unité 567, Paris 75014, France
[3]Centre National de la Recherche Scientifique, Institut de Pharmacologie et de Biologie Structurale (IPBS), Toulouse F-31077, France
[4]Université de Toulouse Université Paul Sabatier, Institute of Pharmacology and Structural Biology, Toulouse F-31077, France
[5]Yale University School of Medicine, New Haven, CT 06520

Junctional adhesion molecules (JAMs) are endothelial and epithelial adhesion molecules involved in the recruitment of circulating leukocytes to inflammatory sites. We show here that JAM-L, a protein related to the JAM family, is restricted to leukocytes and promotes their adhesion to endothelial cells. Cis dimerization of JAM-L is required to engage in heterophilic interactions with its cognate counter-receptor CAR (coxsackie and adenovirus receptor). Interestingly, JAM-L expressed on neutrophils binds CAR independently of integrin activation. However, on resting monocytes and T lymphocytes, which express the integrin VLA-4, JAM-L molecules engage in complexes with VLA-4 and mainly accumulate in their monomeric form. Integrin activation is required for the dissociation of JAM-L–VLA-4 complexes and the accumulation of functional JAM-L dimers, which indicates that the leukocyte integrin VLA-4 controls JAM-L function in cis by controlling its dimerization state. This provides a mechanism through which VLA-4 and JAM-L functions are coordinately regulated, allowing JAM-L to strengthen integrin-dependent adhesion of leukocytes to endothelial cells.

Introduction

Leukocyte migration from blood to tissues plays a central role in inflammatory and immune responses. This migration of leukocytes occurs in a multistep process that involves cognate interactions between circulating cells and the vascular wall (Ley et al., 2007). The first interactive step mediated by selectins results in rolling of the leukocytes along the vascular endothelium. Exposure of leukocytes to chemokines released and presented by inflamed tissues triggers the activation of leukocyte integrins. Activated integrins therefore engage ligands on vascular endothelial cells to mediate firm adhesion of leukocytes to the vessel wall and their movement toward interendothelial junctions (Schenkel et al., 2004). These steps precede the diapedesis of the leukocytes from the vascular lumen into the surrounding tissue by migrating through junctions between endothelial cells, and in some cases, by migrating through the endothelial cell body (Vestweber, 2007).

Several endothelial adhesion molecules act as leukocyte integrin ligands and are involved in leukocyte migration. Leukocyte firm adhesion is mediated by the interaction of endothelial vascular cell adhesion molecule-1 (VCAM-1) with the integrin $\alpha_4\beta_1$ (VLA-4) and of intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2) with the integrins $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1). In addition, the members of the junctional adhesion molecule (JAM) family expressed by endothelial cells have been proposed to constitute other leukocyte integrin ligands (Bazzoni, 2003; Ebnet et al., 2004; Weber et al., 2007). The JAM protein family consists of three members: JAM-A, JAM-B, and JAM-C, which are Ig superfamily molecules with two extracellular Ig domains and a short cytoplasmic tail ending with a type II PDZ-binding motif. The prototypical member of the family, JAM-A, was initially described as a tight junction molecule expressed by endothelial and epithelial cells and involved in monocyte migration in vivo (Martin-Padura et al., 1998).

Correspondence to Sandrine Bourdoulous: sandrine.bourdoulous@inserm.fr

Abbreviations used in this paper: BS[3], bis(sulfosuccinimidyl)suberate; CAR, coxsackie and adenovirus receptor; CMFDA, 5-chloromethylfluorescein diacetate; ESAM, endothelial cell-selective adhesion molecule; HBMEC, human bone marrow endothelial cell; ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; VCAM, vascular cell adhesion molecule.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 183 No. 6 1159–1173
www.jcb.org/cgi/doi/10.1083/jcb.200805061

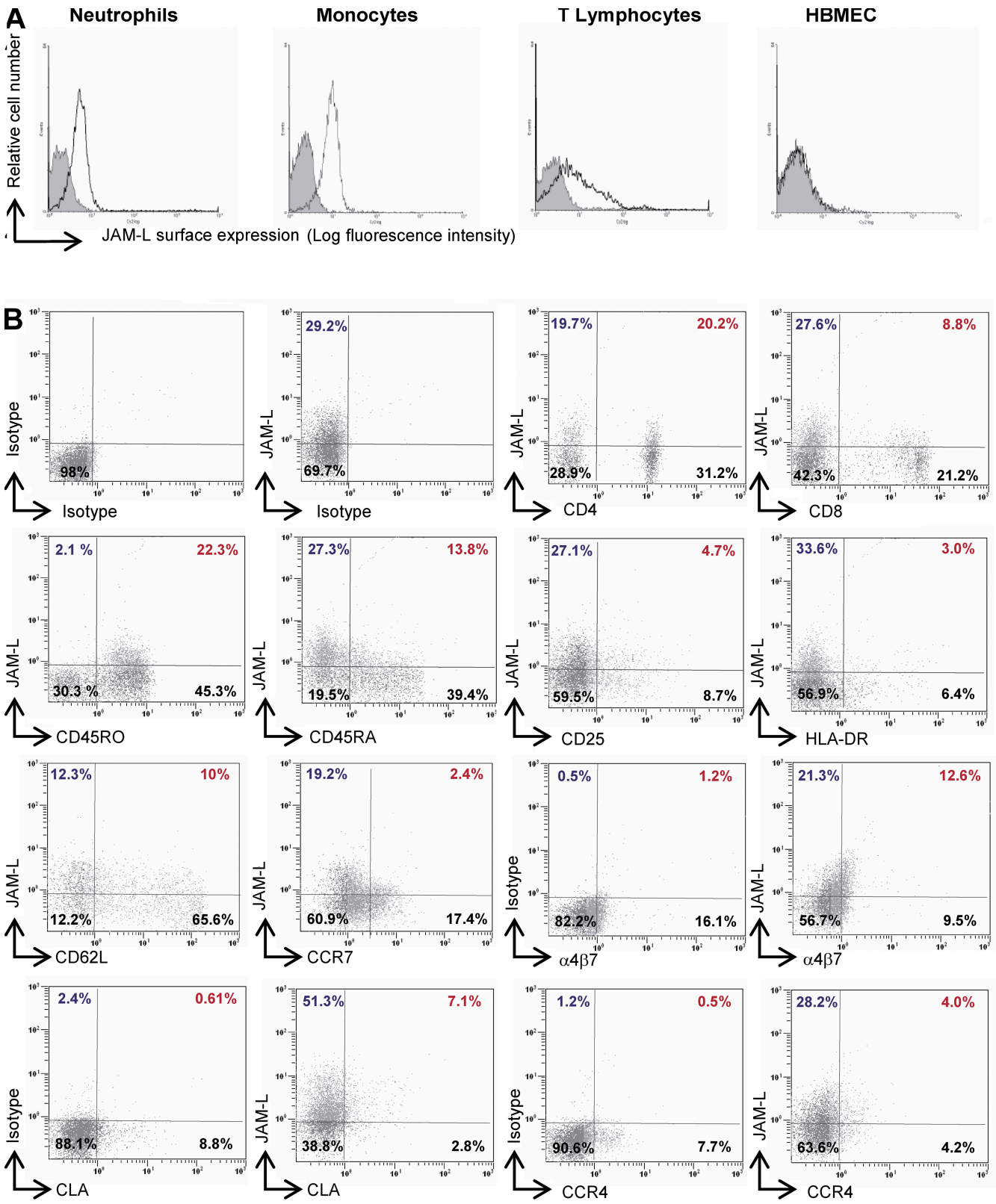

Figure 1. **JAM-L expression is restricted to neutrophils, monocytes, and memory T cells.** (A) Neutrophils, monocytes, and T lymphocytes freshly isolated from human peripheral blood and HBMECs stained with anti–JAM-L and analyzed by flow cytometry. Gray areas represent staining with secondary antibody alone. (B) Freshly isolated human T lymphocytes stained were with antibodies directed against the molecules indicated, fixed, and analyzed by flow cytometry. Lines demarcate the threshold for positive staining for both markers.

JAM-A binds the leukocyte integrin LFA-1 and regulates the adhesion and transmigration of lymphocytes in vitro (Ostermann et al., 2002). In addition, JAM-A is expressed by leukocytes, platelets, and erythrocytes. The two other members of the family, JAM-B and JAM-C, were described as endothelial adhesion molecules highly expressed by high endothelial venules and lymphatic endothelial cells in lymphoid organs, respectively (Cunningham et al., 2000; Palmeri et al., 2000; Arrate et al., 2001; Aurrand-Lions et al., 2001). Endothelial JAM-C can promote lymphocyte migration through homophilic interactions (Johnson-Leger et al., 2002) and is involved in neutrophil transendothelial migration by interacting with Mac-1 (Chavakis et al., 2004). In addition, JAM-C has been shown to be expressed on human leukocytes and to interact with JAM-B (Arrate et al., 2001; Liang et al., 2002), an interaction that facilitates the binding of endothelial JAM-B to the integrin VLA-4 expressed on lymphocytes (Cunningham et al., 2002). Therefore, JAM family members seem to facilitate leukocyte transmigration by interacting in trans with the leukocyte β2 integrins LFA-1 and Mac-1, as well as with the β1 integrin VLA-4, through their extracellular domains, and by engaging in heterophilic interactions in trans among JAM family members.

Additional Ig superfamily members structurally related to the JAMs and potentially involved in leukocyte–endothelial cell interactions have been identified: the coxsackie and adenovirus receptor (CAR), endothelial cell-selective adhesion molecule (ESAM), JAM-4, and JAM-L. CAR, ESAM, and JAM-4 molecules are expressed at endothelial and epithelial junctions and share a similar overall organization with JAMs: two Ig-like domains, a single transmembrane domain, and a longer cytoplasmic tail that ends in a canonical type I PDZ domain–binding sequence. JAM-L, however, is expressed on leukocytes, and its cytoplasmic tail does not possess a PDZ-binding motif (Moog-Lutz et al., 2003). ESAM is involved in the extravasation of neutrophils but not that of lymphocytes (Wegmann et al., 2006), and JAM-L can participate in neutrophil migration across epithelial tight junctions by interacting with CAR (Zen et al., 2005). The molecular mechanisms by which JAM-L and other JAM-related molecules may facilitate the transmigration of specific leukocyte populations are still largely unresolved.

In this study, we found that JAM-L–mediated leukocyte adhesion to endothelial cells is regulated in cis by VLA-4 integrin activation. We provide evidence that JAM-L expressed on neutrophils can directly interact with its cognate counter-receptor CAR independently of integrin activation. However, on resting monocytes and T lymphocytes, which, unlike neutrophils, express the integrin VLA-4, JAM-L constitutively associates with VLA-4. This interaction prevents the cis dimerization of JAM-L, which is required for the functional recognition of CAR. Integrin activation is thus necessary to dissociate JAM-L–VLA-4 complexes and to promote the formation of JAM-L dimers. Our results demonstrate that the leukocyte integrin VLA-4 unexpectedly regulates JAM-L function in cis by controlling its dimerization state; they strongly suggest that VLA-4 integrin and JAM-L functions are coordinately regulated, allowing JAM-L to contribute to the strengthening of the integrin-dependent adhesion of leukocytes to endothelial cells.

Results

JAM-L expression is restricted to neutrophils, monocytes, and memory T cells

We previously identified JAM-L as a novel protein with homology to members of the JAM family. JAM-L mRNA was upregulated in myeloid leukemia cells induced to differentiate through the granulocytic and monocytic pathways, and was expressed in normal hematopoietic cells, including neutrophils, monocytes, and lymphocytes (Moog-Lutz et al., 2003). Using anti–human JAM-L antibodies, we investigated JAM-L expression in freshly isolated human leukocytes and in cultured endothelial or epithelial cells. Consistent with the presence of JAM-L mRNA in leukocytes, JAM-L protein expression was detected at the surface of neutrophils, monocytes, and, to a lesser extent, human T lymphocytes (Fig. 1 A). However, JAM-L expression was not detected on human $CD34^+$ hematopoietic stem cells and progenitors freshly isolated from umbilical cord blood (unpublished data). In addition, neither JAM-L protein (Fig. 1 A) nor mRNA expression (not depicted) were detected in a human bone marrow microvascular endothelial cell line; nor was it detected in endothelial or epithelial cells of different origins (primary cultures of human umbilical vein endothelial cells, saphenous vein endothelial cells, and dermal microvascular endothelial cells, as well as in the brain microvessel endothelial cell line hCMEC/D3 or the epithelial cells lines T84 and Caco-2; not depicted), which strongly suggests that, unlike any other JAMs and related molecules, human JAM-L expression is restricted to mature leukocytes.

Characterization of JAM-L expression among T lymphocyte subpopulations showed that JAM-L is expressed on both $CD4^+$ and $CD8^+$ T cells (~40% and 30%, respectively; Fig. 1 B) with a memory cell phenotype. Staining with antibodies to CD45RA and CD45RO isoforms of the leukocyte common antigen, which functionally identify distinct "naive" and "memory" T cell subsets, respectively, indicates that 90% of the JAM-L–positive cells express $CD45RO^+$, whereas only 30% express CD45RA. In addition, 8–15% express $CD25^+$ and $HLA\text{-}DR^+$, two markers of the activated T cells (Fig. 1 B). JAM-L was therefore mainly expressed on a large subpopulation of memory T cells (30–35% of $CD45RO^+$ T cells). Interestingly, JAM-L expression was predominant on L-selectin and CC-chemokine receptor 7–negative T cells ($CD62L^-$ and $CCR7^-$) that characterize a subpopulation of effector memory T cells, which preferentially migrate to peripheral tissues, as opposed to $CD62L^+$ and $CCR7^+$ central memory T cells, which preferentially migrate to lymph nodes (Lefrancois, 2006). In addition, JAM-L was expressed on a fraction of T cells expressing the integrin α4β7 (associated with gut homing) and of T cells expressing the cutaneous leukocyte antigen (CLA) and CC-chemokine receptor 4 (CCR4; associated with skin homing).

Collectively, these findings indicate that JAM-L is selectively expressed by the first effectors of both innate and adaptative immune responses and strongly suggest that it may directly participate in the trafficking of memory T lymphocytes to nonlymphoid organs.

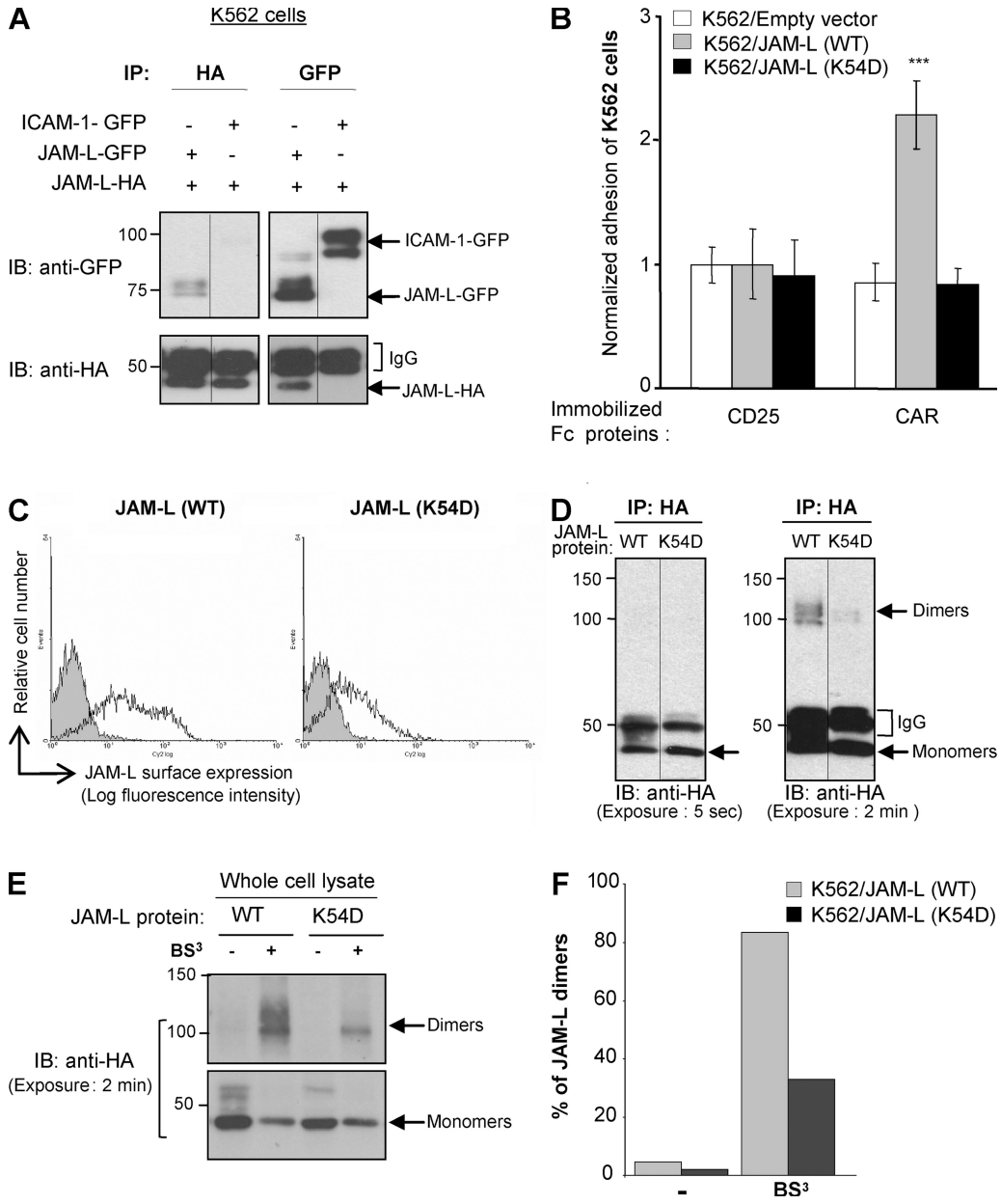

Figure 2. **Cis-dimerization of JAM-L is required for its interaction with CAR.** (A) K562 cells were double transfected with vectors encoding JAM-L–HA and JAM-L–GFP or with JAM-L–HA and ICAM-1–GFP. After lysis, immunoprecipitations (IP) were performed with anti-HA or anti-GFP, as indicated. Immune complexes were subjected to immunoblotting (IB) with anti-HA or anti-GFP. Arrows indicate the electrophoretic mobility of JAM-L–HA, JAM-L–GFP, or ICAM-1–GFP. The bracket indicates an IgG heavy chain. (B) K562 cells were transfected with vectors encoding wild-type (JAM-L–WT, gray) or mutated (JAM-L–K54D, black) JAM-L coupled to HA, or empty vector (white) as control. Cells were then loaded with CMFDA, and adhesion assays were performed on immobilized CAR-Fc or CD25-Fc proteins in RPMI medium. Cells were incubated for 40 min at 37°C, adherent cells were lysed, and fluorescence intensity was measured. The basal adhesion of K562 empty vector cells on CD25-Fc protein was 8% of the total number of incubated cells. Results are means ± SD (n = 4) of a representative three independent experiments (***, $P < 0.001$). (C) K562 cells expressing JAM-L–WT–HA or JAM-L–K54D–HA were labeled using anti–JAM-L, fixed, and analyzed by flow cytometry (bold black lines). Gray areas represent staining with the secondary antibody alone. (D) K562 cells expressing JAM-L–WT–HA or JAM-L–K54D–HA were lysed, then immunoprecipitations and immunoblots were performed with anti-HA. Two exposure times are shown for each blot. Arrows indicate the electrophoretic mobility of monomers and dimers of JAM-L–HA. Black lines indicate that intervening lanes have been spliced out. Numbers next to gel blots indicate molecular mass markers in kD. (E) K562 cells were transfected with JAM-L–WT–HA or JAM-L–K54D–HA and treated or not treated with BS^3 before cell lysis and Western blot analysis with anti-HA. (F) The proportion of dimeric and monomeric forms of JAM-L–WT–HA (gray) or JAM-L–K54D–HA (black) in E was calculated using ImageJ.

Cis dimerization of JAM-L is required to engage in heterophilic interactions with CAR

We previously found that exogenous expression of JAM-L in myeloid cells resulted in enhanced cell adhesion to both resting and cytokine-stimulated human bone marrow endothelial cells (HBMECs; Moog-Lutz et al., 2003). Interestingly, an interaction between JAM-L and epithelial CAR has been described supporting the transepithelial migration of neutrophils (Zen et al., 2005). By expressing JAM-L in the human chronic

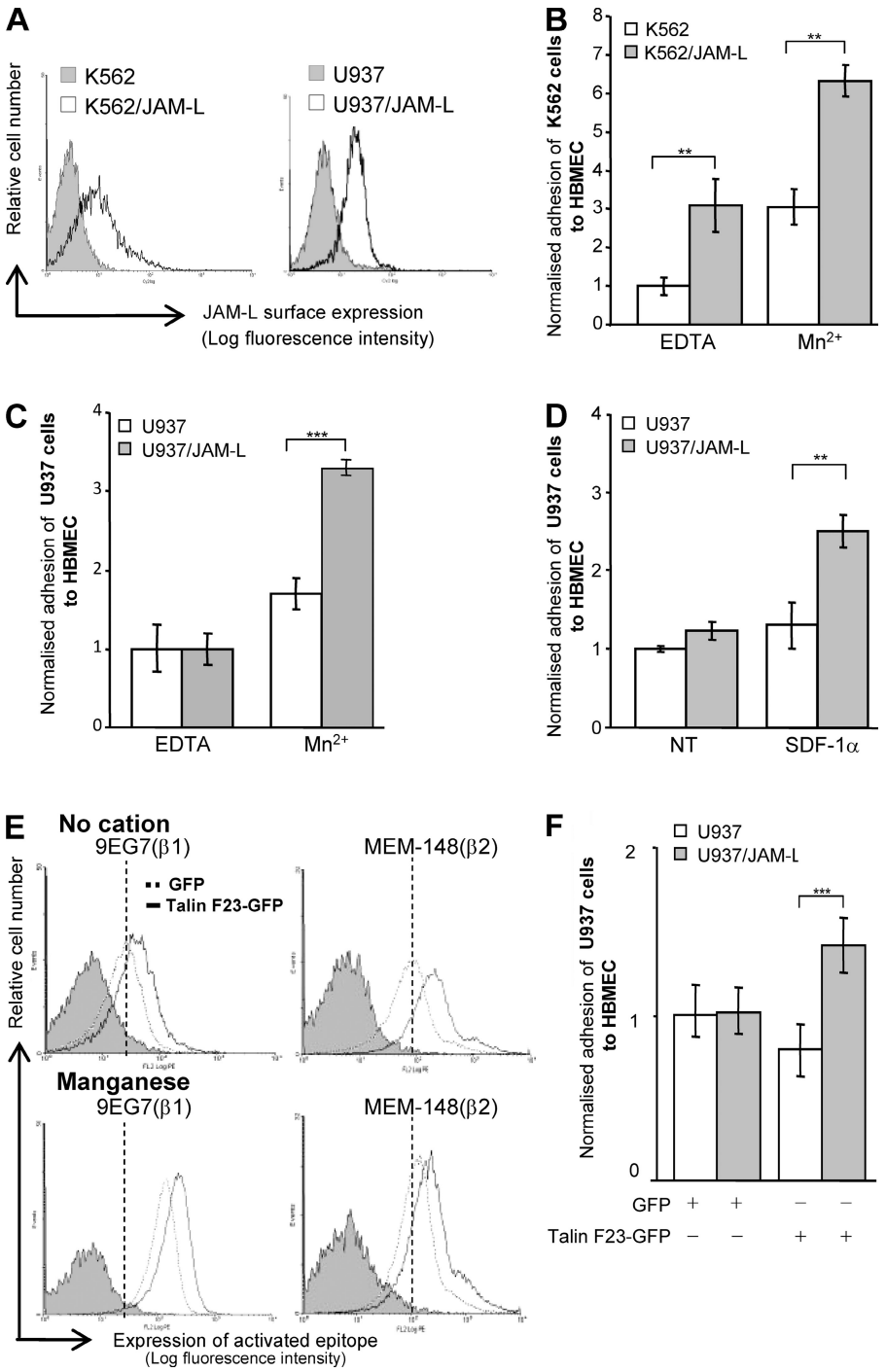

Figure 3. **In monocytic cells, JAM-L function requires integrin activation in cis to enhance cell adhesion.** (A) K562 cells transfected with an empty vector or a vector encoding JAM-L (left) or U937/pMT–JAM-L–HA cells untreated or treated with Zn^{2+} to induce JAM-L–HA expression (right) were labeled using anti–JAM-L, then fixed and analyzed by flow cytometry. (B) K562 cells were transfected with JAM-L–GFP (gray) or GFP alone (white). Cells were preincubated for 5 min at 37°C in PBS with EDTA or Mn^{2+}, and adhesion to HBMECs was assayed in the same buffer for 30 min at 37°C. The number of adherent GFP-positive cells was determined by FACS analysis. The basal adhesion level was 2% of the total number of incubated cells (**, $P < 0.01$). (C and D) U937 cells stably transfected with pMT–JAM-L–HA were untreated (white) or treated (gray) with Zn^{2+} to induce JAM-L expression and then loaded with CMFDA. (C) U937 cells were preincubated for 5 min at 37°C in PBS with EDTA or Mn^{2+}. Adhesion to HBMECs was assayed in the same buffers for 30 min at 37°C. The basal adhesion level was 5% (***, $P < 0.001$). (D) U937 cells were left untreated (NT) or stimulated for 5 min at 37°C with 100 ng/ml SDF-1α in PBS Ca^{-}/Mg^{-}. Cells were then subjected to adhesion to HBMECs in the same buffers for 30 min at 37°C. The basal adhesion level was 5% (**, $P < 0.01$). (E) FACS analysis of U937/pMT–JAM-L–HA transfected with Talin-F23–GFP (bold black lines) or with GFP alone (broken lines). Cells were stained with 9EG7 or MEM-148 antibodies, which recognize active β1 or β2 integrin subunits, respectively, in PBS (no cation, top) or PBS with Mn^{2+} (manganese, bottom). Only GFP-positives cells were analyzed by flow cytometry. Gray areas represent staining profiles with secondary antibodies alone. (F) U937/pMT–JAM-L–HA cells were untreated (white) or treated (gray) with Zn^{2+} and transfected with Talin-F23–GFP or GFP for 8 h. Adhesion to HBMECs was assayed in PBS Ca^{-}/Mg^{-} for 30 min at 37°C. The number of adherent GFP-positive cells was determined by FACS analysis. The basal adhesion level was 6% (***, $P < 0.001$). Results in B, C, D, and F are means ± SD ($n = 3$) of a representative three independent experiments.

myeloid leukemia cell line K562, which lacks endogenous JAM-L, we also observed that these cells adhere specifically to CAR (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200805061/DC1). Additionally, JAM-L–mediated adhesion of K562 cells to HBMECs was prevented by the addition of soluble CAR protein (CAR-Fc chimeric molecules), which is consistent with the idea that JAM-L can promote leukocyte adhesion to endothelial cells by interacting with CAR (Fig. S1).

Crystal structures have revealed that JAM-A molecules form homodimers in cis via a dimerization motif in the membrane distal Ig-like domain (Kostrewa et al., 2001; Prota et al., 2003). Moreover, Cis dimerization of JAM-A was shown to be required for homophilic interaction and function in intercellular junction assembly (Bazzoni et al., 2000; Liu et al., 2000; Mandell et al., 2004). As this dimerization motif is conserved between JAM members and JAM-L, we questioned whether JAM-L forms homodimers in cis. For this purpose, K562 cells were cotransfected with two constructs of JAM-L bearing different tags, JAM-L-GFP and JAM-L-HA, or cotransfected with JAM-L-HA and ICAM-1–GFP, as a control. As shown in Fig. 2 A, JAM-L–GFP was coimmunoprecipitated with JAM-L–HA, and vice versa.

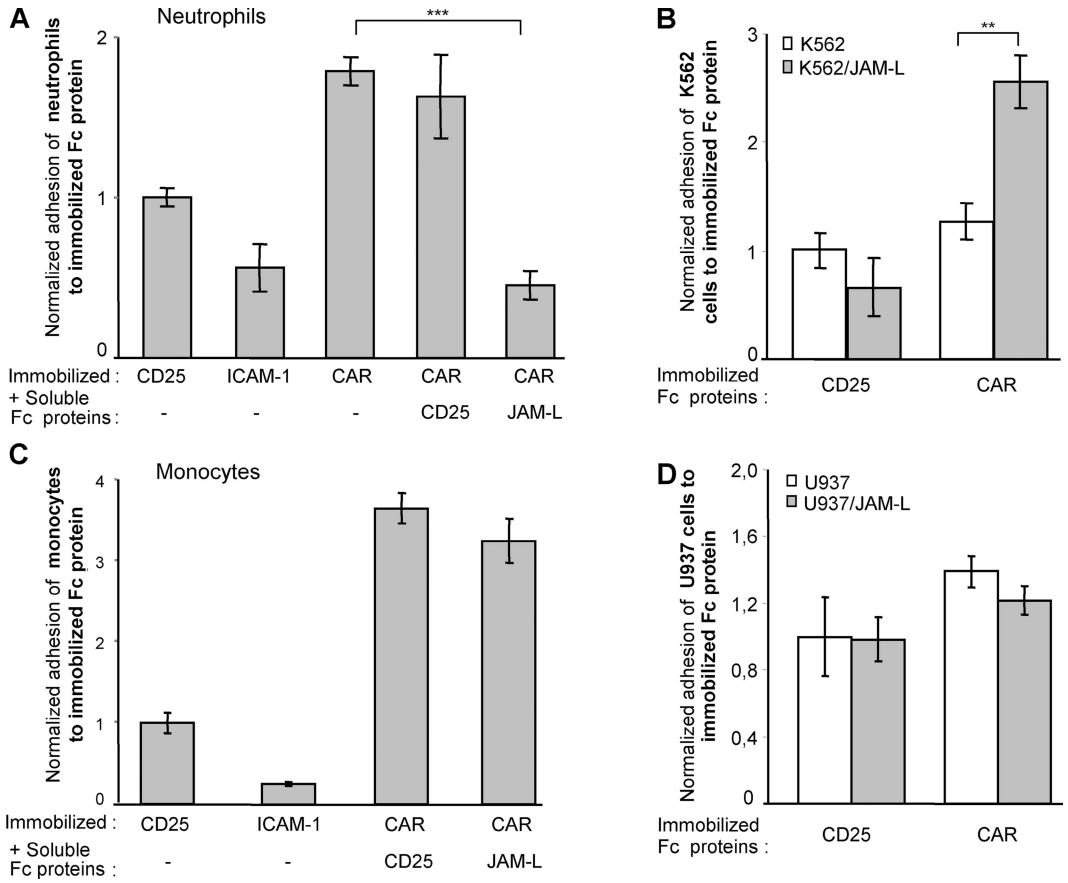

Figure 4. **The dependence of JAM-L–mediated adhesion of human leukocytes on integrin activation is cell type specific.** Freshly isolated human neutrophils (A) or monocytes (C) were loaded with CMFDA, incubated with 20 µg/ml of soluble JAM-L–Fc or CD25-Fc, or no soluble proteins (−), as indicated, for 30 min at 37°C, then added to wells containing immobilized chimeric CD25-Fc, ICAM-1-Fc, or CAR-Fc proteins for 40 min at 37°C in PBS Ca^{-}/Mg^{-} and with EDTA. Adherent cells were lysed and fluorescence intensity was measured. Results are expressed as the amount of fluorescence released from adherent cells, normalized relative to the adhesion of the cells to CD25-Fc, to which 7% of neutrophils or 14% of monocytes adhered. Results are means ± SD (n = 4) of a representative four independent experiments (***, $P < 0.001$). (B) K562 cells were transfected with vectors encoding JAM-L–GFP (gray) or GFP alone (white), and added to wells containing immobilized chimeric CD25-Fc or CAR-Fc proteins for 40 min at 37°C in PBS Ca^{-}/Mg^{-} with EDTA. The number of adherent GFP-positive cells was determined by FACS analysis. The basal adhesion level was 7%. Results are means ± SD (n = 3) of a representative three independent experiments (**, $P < 0.01$). (D) U937 cells stably transfected with pMT–JAM-L–HA were untreated (white) or treated (gray) with Zn^{2+} to induce JAM-L expression. Cells were loaded with CMFDA and added to wells containing immobilized chimeric CD25-Fc or CAR-Fc proteins for 40 min at 37°C in PBS Ca^{-}/Mg^{-} with EDTA. The basal adhesion level was 26%. Results are means ± SD (n = 3) of a representative three independent experiments.

However, no coimmunoprecipitation between JAM-L–HA and ICAM-1–GFP was observed, therefore specifically demonstrating that JAM-L is able to homodimerize.

To assess whether JAM-L homodimer formation was required for JAM-L function, we used a JAM-L construct with a substitution of the lysine 54 by aspartic acid in the dimerization motif (JAM-L^{K54D}). As CAR binding to JAM-L is mediated by an interaction between the membrane-distal Ig domain of CAR and the membrane-proximal Ig domain of JAM-L (Zen et al., 2005), this mutation is unlikely to directly perturb the interaction. We have previously shown that this mutation abolished the ability of JAM-L to promote leukocyte adhesion to an endothelial monolayer when expressed in the monocytic U937 cell line (Moog-Lutz et al., 2003). Using K562 cells transfected with either JAM-L–HA or JAM-L^{K54D}–HA, we show here that this point mutation also abolished JAM-L–mediated adhesion to immobilized CAR-Fc (Fig. 2 B), although both proteins could be detected at the cell plasma membrane by flow cytometry (Fig. 2 C). Because JAM-A was shown to form SDS-resistant dimers (Prota et al., 2003), we analyzed the ability of the K54D point mutation to inhibit JAM-L dimerization by immunoprecipitation and immunoblotting. After immunoprecipitation with an anti-HA antibody, a monomeric form of ∼48 kD was detected in immunocomplexes from K562 cells transfected with JAM-L–HA or JAM-L^{K54D}–HA. In addition, when further exposed, minor bands of ∼100 kD corresponding to dimeric forms of JAM-L were detected from K562 cells expressing JAM-L but were barely visible in the case of JAM-L^{K54D} (Fig. 2 D). Furthermore, using Bis(sulfosuccinimidyl)suberate (BS^3), a noncleavable, membrane-impermeable cross-linking agent, before cell lysis and analysis by Western blotting, we observed that 80–90% of the JAM-L molecules present in K562 cells were stabilized in a dimeric form, whereas this proportion was decreased to 30–35% by the K54D substitution. This, therefore, clearly shows that most of the JAM-L molecules form dimers at the

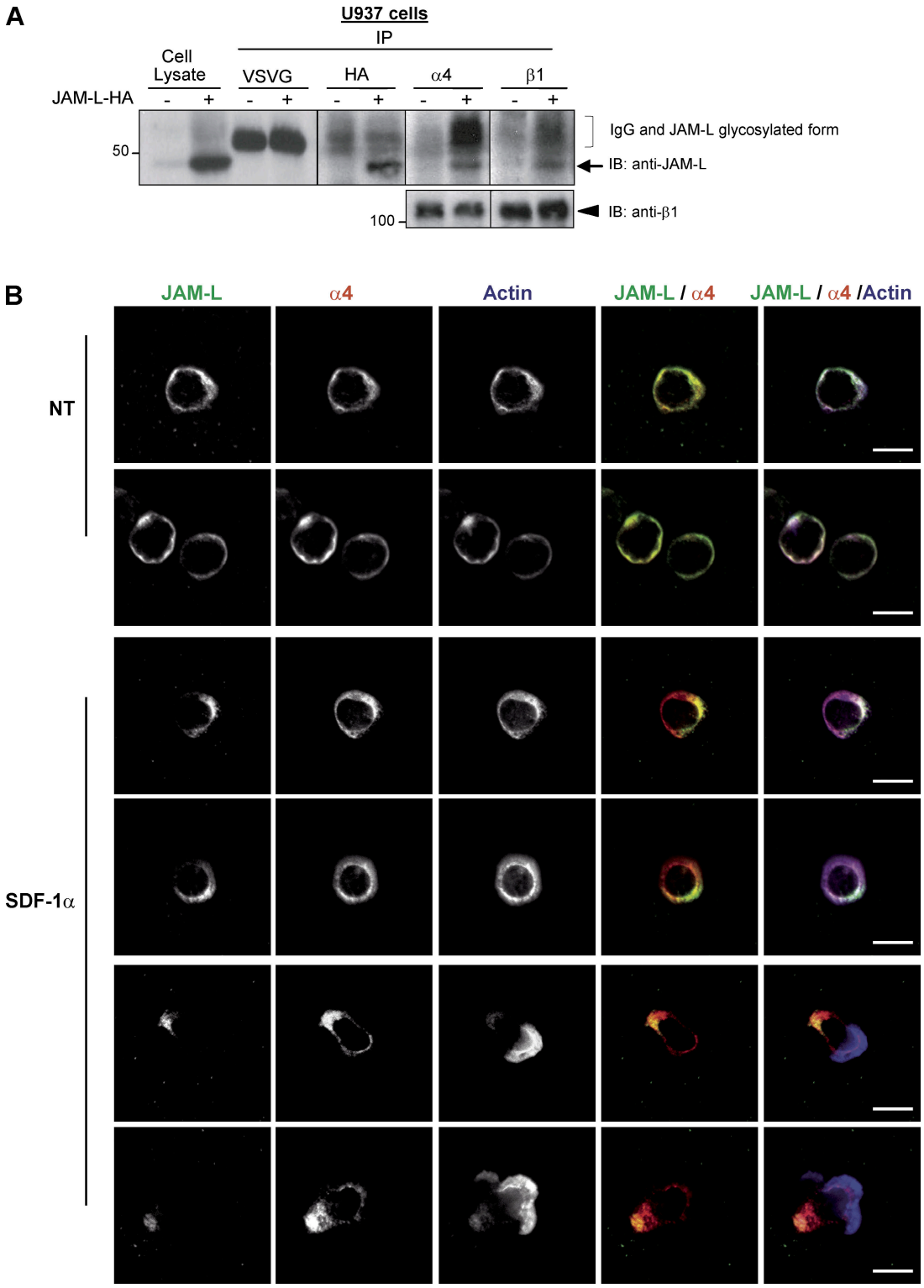

Figure 5. **JAM-L associates with VLA-4.** (A) U937/pMT–JAM-L–HA cells were untreated (−) or treated (+) with Zn^{2+} to induce JAM-L–HA expression. After lysis, immunoprecipitations (IP) were performed with mouse IgG1 isotype antibodies directed against HA, α4, β1, or VSV-g epitope tag as a negative control. Immune complexes were immunoblotted (IB) with anti-HA or anti-β1 antibodies. Black lines indicate that intervening lanes have been spliced out. Numbers next to gel blots indicate molecular mass markers in kD. (B) Freshly isolated T lymphocytes were plated on poly-L-lysine and stimulated or not stimulated for 10 min at 37°C with 100 ng/ml SDF-1α in PBS Ca^{-}/Mg^{-} before fixation. Cells were triple stained for JAM-L (green), α4 subunit (red), and actin (blue), then analyzed by confocal microscopy. (right) Merged images (overlay) of the same fields. Bars, 10 μM.

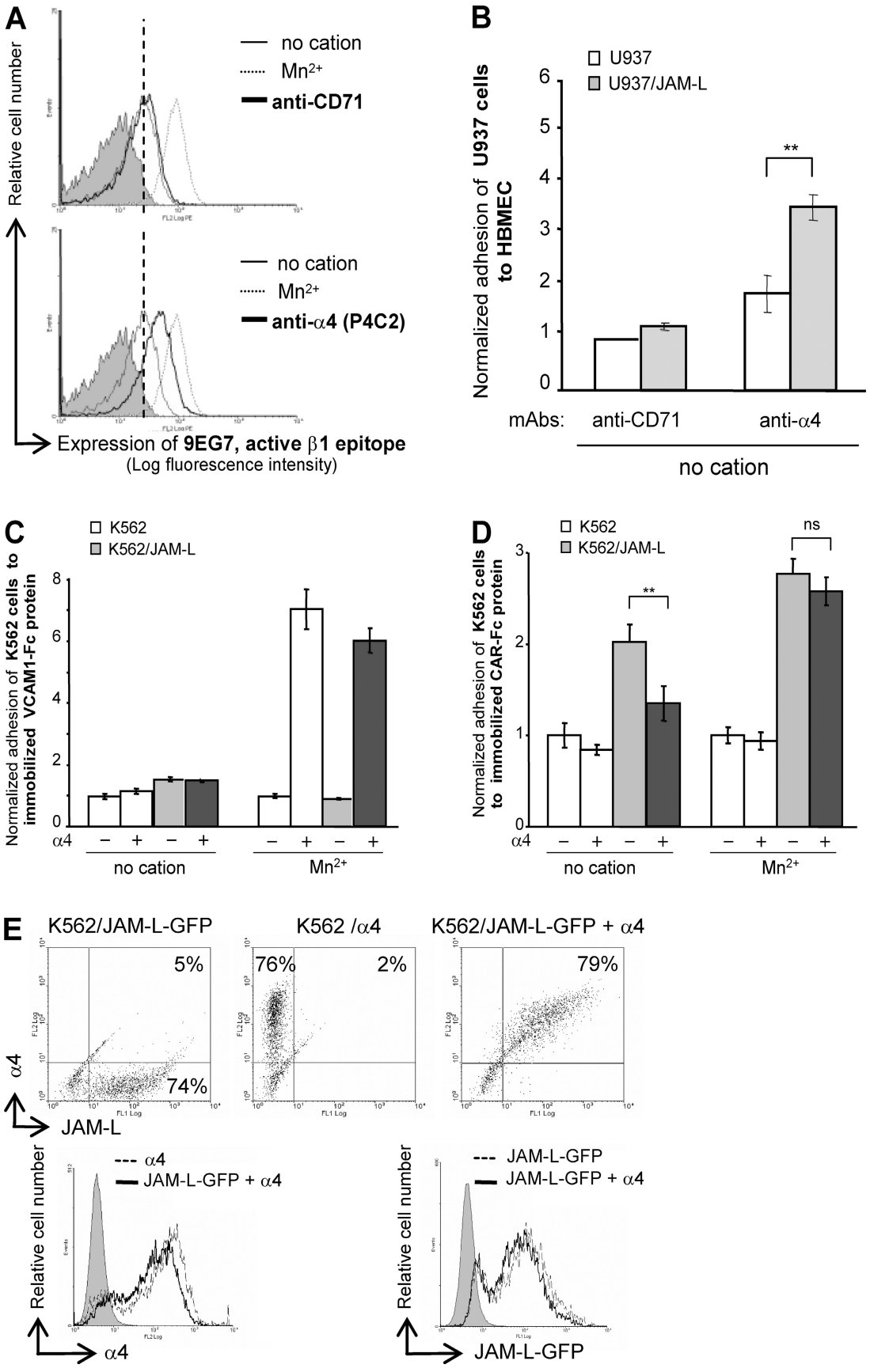

Figure 6. **JAM-L function is regulated in cis by the activation state of VLA-4 integrin.** (A) U937/pMT–JAM-L–HA cells were incubated with antibodies against α4 subunit (P4C2) or CD71 for 30 min at 37°C. Cells were washed and surface stained with 9EG7 antibody at 4°C for 30 min in the absence of cation (bold black lines). In addition, U937 cells were incubated with 9EG7 antibody in the presence of Mn^{2+} (gray broken lines) as a positive control

membrane and that the K54D mutation affects the stabilization of JAM-L dimers. These results provide the first evidence of the importance of dimerization of JAM-related proteins to engage in heterophilic interactions.

JAM-L–mediated adhesion of human monocytes or T lymphocytes, but not that of neutrophils, requires integrin activation

In characterizing the JAM-L–dependent adhesion of leukocytes to endothelial cells, we routinely performed adhesion in RPMI medium containing the cations Ca^{2+}, Mg^{2+}, and Mn^{2+}. The dependence of integrin function on divalent cations is well established (Luo et al., 2007); therefore, we examined the possible role of integrins in JAM-L–mediated adhesion by analyzing the dependence of K562 cells or U937 cells (expressing or not expressing JAM-L to a similar extent; Fig. 3 A) on divalent cations for adhesion to endothelial cells using PBS buffer containing EDTA (which inhibits integrin function) or Mn^{2+} (which activates integrins). Intriguingly, JAM-L–mediated adhesion of K562 cells to HBMECs was observed both in PBS buffer containing EDTA or Mn^{2+} (Fig. 3 B), whereas JAM-L–mediated adhesion of U937 cells, which express zinc-inducible JAM-L–HA, was strictly dependent on the presence of Mn^{2+} (Fig. 3 C), which suggests the involvement of an integrin in JAM-L–mediated function in U937 cells.

To confirm this hypothesis, an adhesion assay of U937 cells was performed in PBS in the presence or absence of the chemokine stromal cell-derived factor-1 (SDF-1α/CXCL12), a well known "inside-out" activating factor of integrins (Kim et al., 2003). As shown in Fig. 3 D, JAM-L–mediated adhesion of U937 cells to HBMECs was detected only in the presence of SDF-1α. We then questioned whether this effect was caused by the activation of monocytic or endothelial integrins. For that purpose, U937 cells that conditionally express JAM-L–HA were further transiently transfected with either GFP alone (control) or with the head domain of talin coupled to GFP (Talin F23–GFP) to directly activate integrins in those cells through an interaction with their β subunit cytoplasmic tails (Tadokoro et al., 2003). As expected, the expression of Talin F23–GFP in U937 cells led to the activation of both β1 and β2 integrins, as assessed by FACS analysis using antibodies directed against activated β integrins (Fig. 3 E). An adhesion assay of these U937 cells was then performed in PBS (Fig. 3 F). JAM-L–mediated adhesion of U937 cells to HBMECs was observed with Talin F23–GFP–expressing cells but not with control cells. These results demonstrate that the activation of a leukocyte integrin (not endothelial integrin) is required to reveal JAM-L function in U937 cells. This effect was not caused by any increase in β1 or β2 integrin expression, nor integrin activation caused by JAM-L expression (unpublished data).

Because previous work indicated that neutrophil adhesion to CAR can occur in a cation-independent manner (Zen et al., 2005), we then analyzed the contribution of the endogenous JAM-L to the adhesion of freshly isolated neutrophils and monocytes to immobilized CAR in PBS containing EDTA. We observed that the adhesion of neutrophils to CAR was abolished in the presence of soluble JAM-L–Fc, whereas the addition of soluble CD25-Fc at the same concentration as a control had no effect (Fig. 4 A). These results indicate that JAM-L can mediate the adhesion of human neutrophils to CAR independently of integrin activation, as observed with K562 cells, in which JAM-L expression promoted their cation-independent adhesion to CAR (Fig. 4 B). However, the addition of soluble JAM-L–Fc did not affect the adhesion of freshly isolated monocytes to CAR (Fig. 4 C) or of T lymphocytes (not depicted), which indicates that JAM-L does not support their adhesion in the absence of integrin activation. Interestingly, these results are similar to what was observed in the monocytic U937 cell line, in which JAM-L expression did not promote adhesion to CAR in PBS containing EDTA (Fig. 4 D).

Collectively, these results provide the unexpected evidence that JAM-L function is regulated in cis by leukocyte integrins, and that this effect is selectively observed in individual leukocyte subpopulations, which suggests that the integrins responsible for JAM-L regulation are expressed by monocytes and T lymphocytes but not by freshly isolated neutrophils or K562 cells.

JAM-L associates with VLA-4 integrin

VLA-4 ($\alpha_4\beta_1$) is one of the main integrins involved in leukocyte interactions with endothelial cells, and is expressed in T lymphocytes and monocytes but not in K562 cells or neutrophils. As VLA-4 expression correlates with the regulation in cis of JAM-L function, we investigated whether JAM-L could interact with VLA-4. Immunoprecipitations were performed from cell lysates of the U937 cells that conditionally express JAM-L–HA. JAM-L–HA was detected both in α_4 and β_1 precipitates (Fig. 5 A). We therefore determined the cell-surface distribution of endogenous VLA-4 and JAM-L on T lymphocytes by confocal immunofluorescence analysis (Fig. 5 B). When plated on poly-L-lysine in PBS buffer, cells remained predominantly round, and both VLA-4 and JAM-L were evenly distributed over the cell surface, where they

for integrin activation or in the absence of cation (thin black lines) to indicate the basal level of integrin activation. Cells were labeled with R-phycoerythrin–conjugated secondary antibodies, then fixed and analyzed by flow cytometry. Gray areas represent staining with the secondary antibody alone. (B) U937/pMT–JAM-L–HA cells were untreated (white) or treated (gray) with Zn^{2+} to induce JAM-L–HA expression. Cells were labeled with CMFDA and incubated with either anti-α4 (P4C2) or anti-CD71, and adhesion to HBMECs prewashed with PBS Ca^{-}/Mg^{-} was assayed for 30 min at 37°C. Adherent cells were lysed and fluorescence intensity was measured. Basal adhesion was 2% of the total number of incubated cells. Results are means ± SD (n = 3) of a representative three independent experiments (**, $P < 0.01$). (C and D) K562 cells were transfected with vectors encoding JAM-L–GFP (gray) or GFP (white). Also, double transfectants were generated with human α4 subunit (+). K562 cells were added to wells containing immobilized VCAM1-Fc proteins (C) or CAR-Fc proteins (D) in PBS without cations or in the presence of Mn^{2+} for 40 min at 37°C. The number of adherent GFP-positive cells was determined by FACS analysis. Results are expressed as the quantity of adherent cells, normalized relative to the basal adhesion of K562/JAM-L and α4-negative cells. The basal adhesion level on CAR-Fc and VCAM1-Fc was 8% and 2% of the total number of incubated cells, respectively. Results are means ± SD (n = 4) of a representative three independent experiments (**, $P < 0.01$). (E) FACS analysis of K562 cells transfected with vectors encoding JAM-L-GFP alone, α4 subunit alone, or double transfected with both constructs. Lines demarcate the threshold for positive staining for both markers.

largely colocalized. Interestingly, after SDF-1α treatment for 10 min, T lymphocytes spread out and, while VLA-4 remained associated with polymerized cortical actin, JAM-L molecules accumulated at one pole of the cells, where they formed a cap structure. In addition, upon SDF-1α treatment, ∼30% of the cells acquired a polarized morphology, with the formation of a large actin-rich lamellipodia at the leading edge and a spherical membrane protrusion budding at the rear, called a "uropod." In these polarized cells, both VLA-4 and JAM-L were present in the uropod; in addition, a fraction of VLA-4, but not of JAM-L, was present in the mid-hind region. These results indicate that JAM-L appears to colocalize with VLA-4 integrin in the absence of activating stimulus, whereas the two proteins partially overlapped upon SDF-1α treatment.

JAM-L function is regulated in cis by VLA-4 integrin

To assess whether the activation of VLA-4 was sufficient to promote JAM-L function, the adhesion to HBMECs of U937 cells that conditionally express JAM-L–HA was assessed in PBS in the presence of P4C2, an antibody against α_4. The addition of P4C2 antibody to U937 cells induced VLA-4 activation, as assessed by FACS analysis using antibodies directed against an activated β_1 integrin subunit (Fig. 6 A). As a control, addition of an isotype-matched irrelevant antibody (anti-CD71, which binds to U937 cells) failed to activate VLA-4. Interestingly, addition of P4C2 antibody revealed JAM-L–mediated adhesion of U937 cells expressing JAM-L to resting HBMECs in the absence of Mn^{2+} (Fig. 6 B). Similar results were obtained using HP2/1, another antibody with VLA-4–activating activity (unpublished data; McGilvray et al., 1997). These results therefore strongly suggest that the activation of VLA-4 is required to unmask JAM-L function in cellular adhesion.

To extend and further validate the finding that VLA-4 interaction with JAM-L controls the JAM-L–mediated adhesion of leukocytes to endothelial cells, we used the K562 cells, as these cells do not express α_4 but express β_1. Transfection of K562 cells with α_4 restored functional VLA-4 heterodimers, enabling the cation-dependent adhesion of K562 cells to immobilized VCAM-1 (Fig. 6 C), which is in line with previous studies (Munoz et al., 1996). We then observed that, although K562 cells expressing JAM-L alone adhere to immobilized CAR-Fc independently of any integrin-activating factor, as expected (Fig. 6 D), the adhesion of K562 cells expressing both JAM-L and VLA-4 to immobilized CAR-Fc was not observed in the absence of cation (Fig. 6 D). The JAM-L–mediated adhesion of K562 cells expressing JAM-L and VLA-4 to CAR was therefore strictly dependent on the presence of Mn^{2+} (Fig. 6 D). As a control, we show that the expression of JAM-L was not affected by α_4 expression, as assessed by flow cytometry (Fig. 6 E). Conversely, JAM-L expression does not significantly affect α4 (or β1) expression, nor the ability of VLA-4 integrin to bind VCAM-1 in both K562 (Fig. 6, C and E) and U937 cells (not depicted). All together, these results demonstrate that, when coexpressed with JAM-L, VLA-4 integrin activation is required to allow the functional interaction between JAM-L and CAR. JAM-L function is therefore regulated in cis by VLA-4 integrin.

VLA-4 integrin controls the dimerization state of JAM-L

Because JAM-L dimerization is required to engage in heterophilic interactions with CAR (Fig. 2), we then investigated whether the interaction between JAM-L and VLA-4 controlled the dimerization state of JAM-L using K562 cells that we transfected with JAM-L–HA alone or together with α4 (Fig. 7 A). We observed that in K562 cells expressing JAM-L-HA alone, SDS-resistant dimers of JAM-L are constitutively present, even in the absence of SDF-1α treatment (Fig. 7 B, middle), which is consistent with our observation that JAM-L expression can promote the adhesion of K562 cells independently of integrin activation. However, when cotransfected with α4-GFP, a constitutive interaction between α4 and the monomeric form of JAM-L–HA was detected, as previously observed in U937 cells (Fig. 5 A). Indeed, α4-GFP was detected in JAM-L–HA immunoprecipitates (Fig. 7 B, top), and, conversely, JAM-L–HA was detected in α4-GFP immunoprecipitates (Fig. 7 C, top). In addition, this interaction was accompanied by a dramatic decrease in the amount of dimeric JAM-L (Fig. 7 B, middle), which is consistent with our observation that coexpression of both JAM-L and VLA-4 prevents JAM-L–mediated adhesion of K562 cells to CAR (Fig. 6 D).

However, upon integrin activation by cell treatment with SDF-1α, the interaction between VLA-4 and JAM-L monomers was largely decreased (Fig. 7, B and C, top), accompanied by the formation of JAM-L dimers (Fig. 7, middle). This is consistent with the observation of a restored JAM-L–mediated adhesion to CAR in the presence of integrin-activating factor (Fig. 6 D). These results demonstrate that the interaction between JAM-L and VLA-4 blocks JAM-L function by preventing the formation or stabilization of JAM-L dimers. Integrin activation leads to the dissociation of JAM-L–VLA-4 complexes, which is required for JAM-L dimerization and recognition of CAR.

To further address how VLA-4 expression affects the dimerization state of JAM-L, we analyzed the proportion of JAM-L dimers formed in K562 cells in the presence or absence of resting or activated VLA-4 integrins using BS^3 before cell lysis and analysis by Western blotting. As shown in Fig. 7 (D and E), in the absence of α4 expression, 80–90% of the JAM-L molecules present in K562 cells were stabilized by BS^3 treatment in a dimeric form. This proportion was not affected by SDF-1α stimulation. However, when coexpressed with α4, the proportion of JAM-L dimers formed was affected, as most of the JAM-L molecules were now accumulated in their monomeric form (65–70%). Integrin activation upon SDF-1α treatment then restored the accumulation of most of the JAM-L molecules in a dimeric form (75–85%). These results provide evidence that the inactive form of VLA-4 controls JAM-L function by preventing the formation of JAM-L dimers at the plasma membrane. However, it is interesting to note that BS^3 treatment did not increase the proportion of α4 associated with JAM-L (unpublished data), which suggests that the integrin may not be in close contact with JAM-L or may be indirectly associated with JAM-L.

All together, our results demonstrate that VLA-4 regulates JAM-L function in cis by controlling its dimerization state. This finding suggests a mechanism through which VLA-4 integrin and JAM-L functions are coordinately regulated, allowing JAM-L

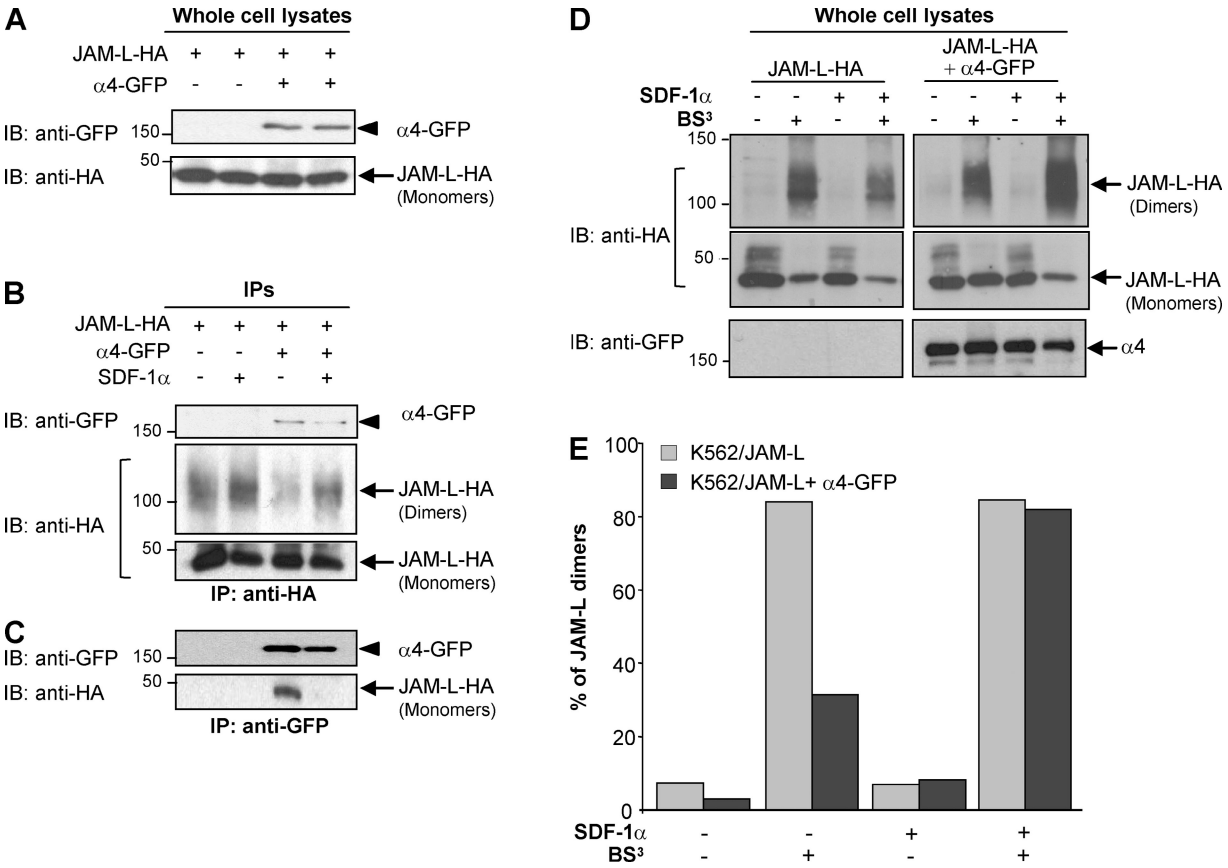

Figure 7. **VLA-4 integrin controls the dimerization state of JAM-L.** (A–C) K562 cells were transfected with JAM-L–HA or double transfected with JAM-L–HA and the human α4 subunit. Cells were then untreated (−) or treated with SDF-1α (100 ng/ml for 5 min) before lysis. (A) Whole cell extracts were analyzed by immunoblotting with anti-HA and anti-GFP to show that similar amounts of JAM-L and α4 were expressed in these different conditions. Arrows indicate the electrophoretic mobility of α4-GFP and of monomers and dimers of JAM-L–HA. Lysates were immunoprecipitated (IP; B) with anti-HA or with anti-GFP (C), and immune complexes were separated by SDS-PAGE and immunoblotted (IB) with anti-HA or anti-GFP, as indicated. (D) K562 cells were transfected with JAM-L–HA or double transfected with JAM-L–HA and the human α4 subunit. Cells were then untreated (−) or treated with SDF-1α (100 ng/ml for 5 min), then treated or not with BS^3 before cell lysis and Western blot analysis. Numbers next to gel blots indicate molecular mass markers in kD. (E) The proportion of dimeric and monomeric forms of JAM-L in D was calculated using ImageJ.

to strengthen the integrin-dependent adhesion of leukocytes to endothelial cells.

Discussion

Leukocyte migration from the blood to tissues involves a series of molecular interactions between leukocyte and endothelial cell adhesion molecules. It is established that the activity of these adhesion molecules must be coordinated in time and space for proper leukocyte diapedesis (Ley et al., 2007). Our results here demonstrate an original regulation in cis of the leukocyte adhesion molecule JAM-L function by the integrin VLA-4. Integrin activation is required to promote JAM-L–mediated leukocyte adhesion to endothelial cells, which suggests that these molecules are coordinately regulated to optimally support the adhesion and extravasation of leukocytes.

JAMs and related molecules are known to play multiple functions. Because of their expression on endothelial and epithelial cells, leukocytes, and platelets, JAMs contribute to adhesive interactions with circulating leukocytes and platelets, tight junction formation in epithelial and endothelial cells during the establishment of cell polarity, growth factor–mediated angiogenesis, and vascular pathologies (Ebnet et al., 2004; Weber et al., 2007). JAM-L is related to the JAM family and has particular structural and functional properties. JAM-L does not possess the canonical PDZ-binding motif present at the C terminus of the other JAM molecules and related members, which is essential for their function in the organization of tight junctions and cell polarity in epithelial cells (Ebnet et al., 2004). Unlike the other JAM family members, we did not find JAM-L expressed in various endothelial or epithelial cells. We present evidence here that JAM-L is expressed on neutrophils and monocytes, which are the first line of host defense against infection by bacterial pathogens. These cells are mobilized within hours to sites of inflammation, where they mediate bactericidal activity. In addition, we show that JAM-L is predominantly expressed on effector memory T cells, negative for the expression of L-selectin and the CC-chemokine receptor 7. This observation is of particular interest because this subset of memory T cells is known to migrate in nonlymphoid and mucosal sites, where they can immediately confront the invading pathogens (Lefrancois, 2006). Moreover, JAM-L is found on T cells expressing specific homing molecules involved in trafficking to the skin and the intestinal mucosa. This cellular distribution strongly suggests a major role

of JAM-L in the recruitment to peripheral tissues of the first effectors of both innate and adaptative immune responses.

Our data suggest that JAM-L promotes leukocyte adhesion to endothelial cells by interacting with CAR. This protein, structurally related to the JAMs and best known as a receptor for coxsackie B and adenoviruses, is mainly expressed in polarized epithelia at the tight junctions (Cohen et al., 2001). Adhesive interactions between CAR and JAM-L were shown to facilitate the transepithelial migration of neutrophils (Zen et al., 2005). However, little is known about the tissue and cellular distribution of CAR in human vasculature. Future studies will determine whether JAM-L interaction with endothelial CAR may facilitate the firm adhesion or transmigration of specific leukocyte populations at specific sites. In addition, our data suggest that CAR may also promote the adhesion of monocytes and T lymphocytes through an interaction with another leukocyte receptor that remains to be identified.

The most striking result of the present study is the observation that JAM-L–mediated adhesion of leukocytes to endothelial cells is regulated in cis by the activation state of the VLA-4 integrin. Until now, evidence has been provided of a functional crosstalk between leukocyte adhesion molecules and integrins, promoting the controlled activation of specific integrins at defined steps during the leukocyte adhesion cascade: E- and L-selectin, which support leukocyte rolling, can induce an intermediate affinity conformation of the integrin LFA-1, allowing it to transiently bind to ICAM-1, and therefore to play a role in leukocyte rolling (Kadono et al., 2002; Chesnutt et al., 2006). CD44-mediated rolling may improve VLA-4 ability to support T cell adhesion under flow (Nandi et al., 2004). In addition, ligation of PECAM-1 on leukocytes can lead to integrin activation and increased expression of $\alpha_6\beta_1$ integrin on the surface of transmigrating neutrophils, enabling neutrophil migration through the endothelial basement membrane (Dangerfield et al., 2002). Conversely, our results provide new evidence that the activation of leukocyte integrin promotes the adhesive function of another leukocyte adhesion molecule: JAM-L.

Interestingly, there is now a growing body of literature documenting coordinated functions between integrins and JAM family members: an interaction in trans between integrins and JAM family members can occur during leukocyte diapedesis, as described for JAM-A and JAM-B, which engage in trans with LFA-1 and VLA-4, respectively (Cunningham et al., 2002; Ostermann et al., 2002). In addition, controlled activation in cis of endothelial or epithelial integrins by JAMs was described during cell migration and growth factor–mediated angiogenesis (Mandell et al., 2005; Naik and Naik, 2006; Mandicourt et al., 2007). We demonstrate here that VLA-4 regulates JAM-L function in cis by controlling its dimerization state. Crystal structures revealed that JAM-A molecules form homodimers in cis via a dimerization motif in their membrane-distal Ig-like domain (Kostrewa et al., 2001; Prota et al., 2003). The presence of a short linker sequence connecting the two Ig-like domains imposes a bent conformation, which is stabilized by hydrogen bonding. As a result, dimers emerge from the cell surface in the shape of an inverted U, enabling tetramer formation with extracellular membrane distal domains of JAMs expressed in trans by the adjacent cell (Weber et al., 2007). The dimerization motif of JAM-A was therefore shown to be required for homophilic interaction and function in intercellular junction assembly (Bazzoni et al., 2000; Liu et al., 2000; Mandell et al., 2004). JAM-L possesses a comparable dimerization motif, which suggests that it may dimerize in a similar way. Accordingly, we show here that a dimerization-defective mutant of JAM-L abolishes JAM-L ability to form dimers and engage in heterophilic interactions with its counter-receptor CAR. These data provide evidence that cis dimerization of JAM-L is required for heterophilic interaction, as the dimerization motif of JAM-L was shown to not be directly involved in heterophilic interaction. Indeed, a previous study documented that JAM-L binding to CAR was mediated by an interaction between the membrane-distal Ig domain of CAR and the membrane-proximal Ig domain of JAM-L (Zen et al., 2005). JAM-L cis dimerization via interaction between both membrane-distal Ig domains might either unmask the CAR binding site within the membrane-proximal Ig domain of JAM-L and/or stabilize the interaction in trans between JAM-L and CAR.

How VLA-4 controls JAM-L dimerization and binding to CAR is still unclear. Our data indicate that, when present in an inactive conformation, VLA-4 is engaged in a complex with JAM-L, preventing the formation of JAM-L dimers. Integrin activation by Mn^{2+} or a chemokine such as SDF-1α, which is known to induce conformational changes within the integrin extracellular domains, is accompanied by the dissociation of JAM-L–VLA-4 complexes and the accumulation of JAM-L dimers at the cell surface. It is therefore tempting to speculate that a direct interaction between the extracellular domains of JAM-L and VLA-4 in its inactive conformation prevents the formation of JAM-L dimers. Interestingly, evidence for direct interaction between JAM and integrins has already been provided, thus strengthening our hypothesis: (1) JAM-A interaction in trans with LFA-1 integrin was shown to be mediated by the open active I domain in the αL subunit of LFA-1, which binds to the membrane-proximal Ig domain of JAM-A on endothelial cells (Fraemohs et al., 2004); and (2) VLA-4 can directly bind in trans to the distal Ig domain of JAM-B on endothelial cells (Cunningham et al., 2002). However, no stabilization of VLA-4–JAM-L complexes was found in the presence of the chemical crosslinker, BS^3, which suggests that VLA-4 may not be in close contact with JAM-L. It is therefore possible that VLA-4 might also indirectly affect the dimerization state of JAM-L either through an interaction with other transmembrane proteins or through an interaction with the cortical actin cytoskeleton. Indeed, because chemokine-induced integrin activation promotes specific interactions of the α and β cytoplasmic domains of integrins with components of the cytoskeleton, VLA-4 activation could contribute to the stabilization of functional JAM-L dimers by facilitating its interaction with cytoskeletal regulators. Future structural and biochemical studies will aim to identify the precise mechanisms by which VLA-4 controls JAM-L dimerization.

In addition, it is interesting to note that SDF-1α treatment of K562 cells expressing JAM-L slightly increased the quantity of JAM-L dimers and JAM-L–mediated adhesion to CAR, although these cells do not express VLA-4. Therefore, we cannot exclude the possibility that another integrin may control JAM-L

function to a lesser extent. In light of our present findings, it will now be of particular interest to address whether VLA-4 or other integrins control the functional activity of other adhesion molecules, and, in particular, whether VLA-4 may also control JAM-C function, as the interaction in trans between leukocyte VLA-4 and endothelial JAM-B cannot be observed in the absence of leukocyte JAM-C expression (Cunningham et al., 2002).

In conclusion, by demonstrating an original regulation in cis of the leukocyte adhesion molecule JAM-L function by the integrin VLA-4, our results highlight a new degree of complexity in the post-adhesion events that strengthen leukocyte attachment to the endothelium. Although a network of adhesion molecules with overlapping functions mediate the capture, rolling, and firm adhesion of leukocytes to the vascular endothelium, this study adds a novel twist to this adhesion cascade.

Materials and methods

Antibodies, reagents, and recombinant chimeric Fc-proteins

The anti–human JAM-L (AMICA) antibody was obtained from R&D Systems; anti–integrin α4 (clone P4C2) and β1 (clone P5D2) were obtained from Millipore; anti-β2 activation epitope antibody (clone MEM-148) was obtained from AbD Serotec; anti–integrin β1 activation epitope antibody (clone 9EG7), R-phycoerythrin–conjugated anti-α4β7 antibody, and fluorescent-conjugated anti-CD4, anti-CD8, anti-CD45RO, anti-CD45RA, anti–cutaneous leukocyte antigen (CLA), anti-CCR7, anti-CD25, and anti–CC-chemokine receptor 4 (CCR4) were obtained from BD; anti-CD71 (clone DF1513) was obtained from Santa Cruz Biotechnology, Inc.; anti-HA (clone 12CA5), anti-GFP, and VSV-g epitope tag (clone P5D4) antibodies were obtained from Roche; fluorescent-conjugated secondary antibodies, peroxidase-conjugated secondary antibodies, and ECL reagents were obtained from Jackson ImmunoResearch Laboratories; Alexa Fluor 488–conjugated Phalloidin and the fluorescent cell tracker 5-chloromethylfluorescein diacetate (CMFDA) were obtained from Invitrogen; human recombinant SDF-1α was obtained from Abcys; digitonin and poly-L-Lysine solution were obtained from Sigma-Aldrich; recombinant Fc-chimeric proteins were all purchased from R&D Systems; and BS^3 was obtained from Thermo Fisher Scientific.

Cells and culture conditions

HBMECs, provided by B.B. Weksler (Weill Medical College of Cornell University, New York, NY; Schweitzer et al., 1997), and a U937 human monocytic cell line stably transfected with the pMT-JAML-HA expression vector encoding full-length JAM-L protein fused with a HA epitope (U937/JAM-L) were grown as described previously (Hoffmann et al., 2001; Moog-Lutz et al., 2003). JAM-L protein expression was induced by addition of 0.1 mM of $ZnSO_4$ for 18 h in RPMI culture medium. Human K562 cells from American Type Culture Collection were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 10 mM Hepes. Human leukocytes from peripheral blood of healthy volunteers were isolated by centrifugation through a Ficoll-Paque Plus (GE Healthcare) gradient. Granulocytes were enriched using standard protocols (Doulet et al., 2006); T lymphocytes and monocytes were isolated by magnetic negative cell sorting using a Dynal T Cell Negative Isolation kit and a Dynal Monocyte Negative Isolation kit (Invitrogen). Human $CD34^+$ hematopoietic stem cells were provided by S. Fichelson (Institut Cochin, Paris, France) and directly used for FACS analysis.

cDNA constructs and cell transfections

Vectors encoding JAM-L tagged with the HA epitope at its C terminus (p513-JAM-L-HA vector) and the p513-JAM-L-K54D-HA vector with a mutation in the putative dimerization domain have been described previously (Moog-Lutz et al., 2003). The sequence encoding JAM-L was subcloned into the pEGFP-N3 vector (Clontech Laboratories, Inc.). The vector encoding the head domain of talin coupled to GFP has been described previously (Tadokoro et al., 2003). The vector encoding GFP-tagged human ICAM-1 was provided by F. Sanchez-Madrid (Hospital Universitario de La Princesa, Madrid, Spain). Vectors encoding human nontagged and GFP-tagged α4 integrin were provided by M.J. Humphries (University of Manchester, Manchester, England, UK). Cells were transfected using the Nucleofector system (Amaxa Biosystems). 5×10^6 cells were suspended in 100 µl of Nucleofector solution mix with 2–5 µg of plasmids and subjected to electroporation using the cell line–specific programs: T-16 for K562 cells and V-01 for U937 cells. Cells were then diluted to 0.5×10^6 cells/ml in culture medium for 8 or 24 h before the experiment.

Flow cytometry

Cell surface labeling with nonconjugated antibodies (anti-JAM-L and anti-α4) was performed by sequential incubation of 5×10^5 cells with primary antibodies for 30 min at 4°C in cold FACS buffer (PBS, 3% FCS, and 0.01% sodium azide), followed by incubation of the cells with conjugated secondary antibody for 30 min at 4°C in cold FACS buffer. Cell surface labeling with fluorescent-conjugated antibodies was performed by incubation of 5×10^5 cells with coupled antibodies for 30 min at 4°C in cold FACS buffer. After three washes with FACS buffer, cells were fixed with 4% PFA and analyzed by flow cytometry (FC500 flow cytometer; Beckman Coulter).

Binding of leukocytes to recombinant chimeric-Fc proteins

Purified recombinant Fc chimeric proteins were immobilized overnight at 4°C in 48-well (1 µg/well) or 96-well plates (0.5 µg/well) in 25 mM Tris, pH 8, buffer supplemented with 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. Wells were then saturated with 1% BSA in PBS for 1 h at room temperature. Before binding assays, freshly isolated leukocytes or cell lines were loaded with CMFDA according to the manufacturer instructions. Cells were then washed and diluted in the indicated buffers: either RPMI medium, PBS buffer without Ca/Mg (PBS−/−) supplemented with EDTA, or PBS (−/−) buffer supplemented with 0.5 mM Mn^{2+}. Neutrophils or monocytes were added to the coated 48-well plates at 5×10^5 cells per well. K562 cells or U937/JAM-L cells were added to the coated 96-well plates at 2×10^5 cells per well. Cells were incubated for 40 min at 37°C. After three washes in PBS (−/−) buffer supplemented with EDTA or Mn^{2+} to remove nonadherent cells, adherent CMFDA-loaded cells were lysed with water, and fluorescence intensity was quantified using a fluorometer (Fusion; PerkinElmer). Where indicated, K562 cells were transfected with vector encoding JAM-L–GFP, and the number of adherent GFP-positive cells was determined by flow cytometry.

Leukocyte adhesion to HBMEC

HBMEC were seeded on 48-well plates (10^5 cells per well) and cultured for 48 h to reach confluence. Leukocytes were loaded with CMFDA, and adhesion to HBMEC was assayed using 2×10^5 cells per well in PBS (−/−) buffer supplemented with 0.5 mM EDTA, 100 ng/ml SDF-1α, or 0.5 mM Mn^{2+} for 30 min at 37°C. After three washes, adherent cells were lysed with water, and fluorescence intensity was quantified using a fluorometer (Fusion). Where indicated, K562 cells were transfected with vector encoding JAM-L–GFP, and the number of adherent GFP positive cells was determined by flow cytometry. Data are presented as the mean ± SD and were compared by a student's *t* test.

Immunofluorescence microscopy

13-mm Permanox coverslips were coated with poly-L-lysine in PBS buffer for 30 min at 37°C and washed twice with PBS buffer. Freshly isolated T lymphocytes were added in RPMI medium for 30 min at 37°C (5×10^5 per well). Nonadherent cells were removed, and adherent cells were untreated or treated with 100 ng/ml SDF-1α for 10 min at 37°C. Cells were fixed in 4% PFA for 10 min and washed three times with PBS buffer. After a 30-min treatment with blocking solution (PBS and 3% BSA), cells were stained for 2 h with the primary antibodies. After three washes with PBS, cells were incubated for 45 min with a mixture of secondary antibodies coupled to cyanin-2 and cyanin-5, and with rhodamine-phalloidin. Labeled preparations were mounted in Mowiol and were analyzed by confocal microscopy (TCS SP2 AOBS; Leica) with an inverted microscope (DMIRE2; Leica) using a 63× 1.4 NA DIC oil HCX Plan-Apo objective (Leica). Acquisitions were made with a LCS (Leica) and FCS (ISS) software at zoom 6.

Immunoprecipitations, SDS-PAGE, and Western blotting

Leukocytes (5×10^6 cells) were washed with PBS (−/−) buffer. Cells were untreated or treated with 100 ng/ml SDF-1α for 5 min in PBS (−/−) buffer. When indicated, cells were treated with 2 mM BS^3 for 40 min at 4°C, then incubated in 15 mM Tris, pH 7.5, for 15 min at 4°C, according to the manufacturer's instructions, before cell lysis. Cells were then lysed in a digitonin-based buffer (1× TBS, 1% digitonin, 5 mM NaF, 2 mM Na_3VO_4, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride [AEBSF], 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin). The insoluble fractions were removed by centrifugation, and the cleared lysates were used for immunoprecipitations with specific antibodies for 3 h at 4°C. Then, protein G–sepharose beads (GE Healthcare) were added for 1 h at 4°C. Precipitated proteins were washed three times, solubilized in Laemmli buffer, and boiled at 100°C for 5 min. Samples were resolved on 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher & Schuell). After saturation in PBS containing 3% nonfat dry milk and 0.1% Tween 20 (blocking buffer) for 30 min, membranes

were blotted overnight with the indicated antibodies diluted in the blocking buffer. Antibodies were detected with peroxidase-coupled secondary antibodies using the ECL system. Quantification histograms were obtained with ImageJ software (National Institutes of Health).

Online supplemental material

Fig. S1 shows that JAM-L promotes leukocyte adhesion to endothelial cells by interacting with CAR. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200805061/DC1.

We thank B.B. Weksler, S. Fichelson, M.J. Humphries, and F. Sanchez-Madrid for reagents; P. Bourdoncle for technical assistance; and F. Niedergang, N. Simpson, MP. Laran-Chich, J. Delon, and E. Donnadieu for helpful discussions.

This work was supported by the Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, the Université Paris-Descartes, and the Fondation de France. A.-C. Luissint was supported by the Ministère de la Recherche and by Association pour la Recherche sur le Cancer. D.A. Calderwood is supported by the National Institutes of Health.

Submitted: 13 May 2008
Accepted: 12 November 2008

References

Arrate, M.P., J.M. Rodriguez, T.M. Tran, T.A. Brock, and S.A. Cunningham. 2001. Cloning of human junctional adhesion molecule 3 (JAM3) and its identification as the JAM2 counter-receptor. *J. Biol. Chem.* 276:45826–45832.

Aurrand-Lions, M., L. Duncan, C. Ballestrem, and B.A. Imhof. 2001. JAM-2, a novel immunoglobulin superfamily molecule, expressed by endothelial and lymphatic cells. *J. Biol. Chem.* 276:2733–2741.

Bazzoni, G. 2003. The JAM family of junctional adhesion molecules. *Curr. Opin. Cell Biol.* 15:525–530.

Bazzoni, G., O.M. Martinez-Estrada, F. Orsenigo, M. Cordenonsi, S. Citi, and E. Dejana. 2000. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *J. Biol. Chem.* 275:20520–20526.

Chavakis, T., T. Keiper, R. Matz-Westphal, K. Hersemeyer, U.J. Sachs, P.P. Nawroth, K.T. Preissner, and S. Santoso. 2004. The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. *J. Biol. Chem.* 279:55602–55608.

Chesnutt, B.C., D.F. Smith, N.A. Raffler, M.L. Smith, E.J. White, and K. Ley. 2006. Induction of LFA-1-dependent neutrophil rolling on ICAM-1 by engagement of E-selectin. *Microcirculation.* 13:99–109.

Cohen, C.J., J.T. Shieh, R.J. Pickles, T. Okegawa, J.T. Hsieh, and J.M. Bergelson. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc. Natl. Acad. Sci. USA.* 98:15191–15196.

Cunningham, S.A., M.P. Arrate, J.M. Rodriguez, R.J. Bjercke, P. Vanderslice, A.P. Morris, and T.A. Brock. 2000. A novel protein with homology to the junctional adhesion molecule. Characterization of leukocyte interactions. *J. Biol. Chem.* 275:34750–34756.

Cunningham, S.A., J.M. Rodriguez, M.P. Arrate, T.M. Tran, and T.A. Brock. 2002. JAM2 interacts with alpha4beta1. Facilitation by JAM3. *J. Biol. Chem.* 277:27589–27592.

Dangerfield, J., K.Y. Larbi, M.T. Huang, A. Dewar, and S. Nourshargh. 2002. PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate leukocyte migration through the perivascular basement membrane. *J. Exp. Med.* 196:1201–1211.

Doulet, N., E. Donnadieu, M.P. Laran-Chich, F. Niedergang, X. Nassif, P.O. Couraud, and S. Bourdoulous. 2006. *Neisseria meningitidis* infection of human endothelial cells interferes with leukocyte transmigration by preventing the formation of endothelial docking structures. *J. Cell Biol.* 173:627–637.

Ebnet, K., A. Suzuki, S. Ohno, and D. Vestweber. 2004. Junctional adhesion molecules (JAMs): more molecules with dual functions? *J. Cell Sci.* 117:19–29.

Fraemohs, L., R.R. Koenen, G. Ostermann, B. Heinemann, and C. Weber. 2004. The functional interaction of the beta 2 integrin lymphocyte function-associated antigen-1 with junctional adhesion molecule-A is mediated by the I domain. *J. Immunol.* 173:6259–6264.

Hoffmann, I., E. Eugene, X. Nassif, P.O. Couraud, and S. Bourdoulous. 2001. Activation of ErbB2 receptor tyrosine kinase supports invasion of endothelial cells by *Neisseria meningitidis*. *J. Cell Biol.* 155: 133–143.

Johnson-Leger, C.A., M. Aurrand-Lions, N. Beltraminelli, N. Fasel, and B.A. Imhof. 2002. Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. *Blood.* 100:2479–2486.

Kadono, T., G.M. Venturi, D.A. Steeber, and T.F. Tedder. 2002. Leukocyte rolling velocities and migration are optimized by cooperative L-selectin and intercellular adhesion molecule-1 functions. *J. Immunol.* 169:4542–4550.

Kim, M., C.V. Carman, and T.A. Springer. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science.* 301:1720–1725.

Kostrewa, D., M. Brockhaus, A. D'Arcy, G.E. Dale, P. Nelboeck, G. Schmid, F. Mueller, G. Bazzoni, E. Dejana, T. Bartfai, et al. 2001. X-ray structure of junctional adhesion molecule: structural basis for homophilic adhesion via a novel dimerization motif. *EMBO J.* 20:4391–4398.

Lefrancois, L. 2006. Development, trafficking, and function of memory T-cell subsets. *Immunol. Rev.* 211:93–103.

Ley, K., C. Laudanna, M.I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7:678–689.

Liang, T.W., H.H. Chiu, A. Gurney, A. Sidle, D.B. Tumas, P. Schow, J. Foster, T. Klassen, K. Dennis, R.A. DeMarco, et al. 2002. Vascular endothelial-junctional adhesion molecule (VE-JAM)/JAM 2 interacts with T, NK, and dendritic cells through JAM 3. *J. Immunol.* 168:1618–1626.

Liu, Y., A. Nusrat, F.J. Schnell, T.A. Reaves, S. Walsh, M. Pochet, and C.A. Parkos. 2000. Human junction adhesion molecule regulates tight junction resealing in epithelia. *J. Cell Sci.* 113(Pt 13):2363–2374.

Luo, B.H., C.V. Carman, and T.A. Springer. 2007. Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* 25:619–647.

Mandell, K.J., I.C. McCall, and C.A. Parkos. 2004. Involvement of the junctional adhesion molecule-1 (JAM1) homodimer interface in regulation of epithelial barrier function. *J. Biol. Chem.* 279:16254–16262.

Mandell, K.J., B.A. Babbin, A. Nusrat, and C.A. Parkos. 2005. Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on beta1 integrins and Rap1 activity. *J. Biol. Chem.* 280:11665–11674.

Mandicourt, G., S. Iden, K. Ebnet, M. Aurrand-Lions, and B.A. Imhof. 2007. JAM-C regulates tight junctions and integrin-mediated cell adhesion and migration. *J. Biol. Chem.* 282:1830–1837.

Martin-Padura, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, et al. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J. Cell Biol.* 142:117–127.

McGilvray, I.D., Z. Lu, R. Bitar, A.P. Dackiw, C.J. Davreux, and O.D. Rotstein. 1997. VLA-4 integrin cross-linking on human monocytic THP-1 cells induces tissue factor expression by a mechanism involving mitogen-activated protein kinase. *J. Biol. Chem.* 272:10287–10294.

Moog-Lutz, C., F. Cave-Riant, F.C. Guibal, M.A. Breau, Y. Di Gioia, P.O. Couraud, Y.E. Cayre, S. Bourdoulous, and P.G. Lutz. 2003. JAML, a novel protein with characteristics of a junctional adhesion molecule, is induced during differentiation of myeloid leukemia cells. *Blood.* 102:3371–3378.

Munoz, M., J. Serrador, F. Sanchez-Madrid, and J. Teixido. 1996. A region of the integrin VLA alpha 4 subunit involved in homotypic cell aggregation and in fibronectin but not vascular cell adhesion molecule-1 binding. *J. Biol. Chem.* 271:2696–2702.

Naik, M.U., and U.P. Naik. 2006. Junctional adhesion molecule-A-induced endothelial cell migration on vitronectin is integrin alpha v beta 3 specific. *J. Cell Sci.* 119:490–499.

Nandi, A., P. Estess, and M. Siegelman. 2004. Bimolecular complex between rolling and firm adhesion receptors required for cell arrest; CD44 association with VLA-4 in T cell extravasation. *Immunity.* 20:455–465.

Ostermann, G., K.S. Weber, A. Zernecke, A. Schroder, and C. Weber. 2002. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat. Immunol.* 3:151–158.

Palmeri, D., A. van Zante, C.C. Huang, S. Hemmerich, and S.D. Rosen. 2000. Vascular endothelial junction-associated molecule, a novel member of the immunoglobulin superfamily, is localized to intercellular boundaries of endothelial cells. *J. Biol. Chem.* 275:19139–19145.

Prota, A.E., J.A. Campbell, P. Schelling, J.C. Forrest, M.J. Watson, T.R. Peters, M. Aurrand-Lions, B.A. Imhof, T.S. Dermody, and T. Stehle. 2003. Crystal structure of human junctional adhesion molecule 1: implications for reovirus binding. *Proc. Natl. Acad. Sci. USA.* 100:5366–5371.

Schenkel, A.R., Z. Mamdouh, and W.A. Muller. 2004. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat. Immunol.* 5:393–400.

Schweitzer, K.M., P. Vicart, C. Delouis, D. Paulin, A.M. Drager, M.M. Langenhuijsen, and B.B. Weksler. 1997. Characterization of a newly established human bone marrow endothelial cell line: distinct adhesive

properties for hematopoietic progenitors compared with human umbilical vein endothelial cells. *Lab. Invest.* 76:25–36.

Tadokoro, S., S.J. Shattil, K. Eto, V. Tai, R.C. Liddington, J.M. de Pereda, M.H. Ginsberg, and D.A. Calderwood. 2003. Talin binding to integrin beta tails: a final common step in integrin activation. *Science.* 302:103–106.

Vestweber, D. 2007. Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium. *Immunol. Rev.* 218:178–196.

Weber, C., L. Fraemohs, and E. Dejana. 2007. The role of junctional adhesion molecules in vascular inflammation. *Nat. Rev. Immunol.* 7:467–477.

Wegmann, F., B. Petri, A.G. Khandoga, C. Moser, A. Khandoga, S. Volkery, H. Li, I. Nasdala, O. Brandau, R. Fassler, et al. 2006. ESAM supports neutrophil extravasation, activation of Rho, and VEGF-induced vascular permeability. *J. Exp. Med.* 203:1671–1677.

Zen, K., Y. Liu, I.C. McCall, T. Wu, W. Lee, B.A. Babbin, A. Nusrat, and C.A. Parkos. 2005. Neutrophil migration across tight junctions is mediated by adhesive interactions between epithelial coxsackie and adenovirus receptor and a junctional adhesion molecule-like protein on neutrophils. *Mol. Biol. Cell.* 16:2694–2703.