Docking onto the endothelium: Trio directs leukocyte extravasation
van Rijssel, J.

Citation for published version (APA):
van Rijssel, J. (2011). Docking onto the endothelium: Trio directs leukocyte extravasation

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
FILAMIN B MEDIATES ICAM-1-DRIVEN LEUKOCYTE TRANS-ENDOTHELIAL MIGRATION

Edwin Kanters1, Jos van Rijssel1, Paul J. Hensbergen2, David Hondius1, Frederik. P. J. Mul1, André M. Deelder2, Arnoud Sonnenberg3, Jaap D. van Buul1 and Peter L. Hordijk1

1Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands.
2Biomolecular Mass Spectrometry Unit, Department of Parasitology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands.
3Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

ABSTRACT

During inflammation, the endothelium mediates rolling and firm adhesion of activated leukocytes. Integrin-mediated adhesion to endothelial ligands of the Ig-superfamily induces intracellular signalling in endothelial cells which promotes leukocyte transendothelial migration. We identified the actin-crosslinking molecule filamin B as a novel binding partner for Intracellular Adhesion Molecule-1 (ICAM-1). Immune-precipitation as well as laser scanning confocal microscopy confirmed the specific interaction and co-localization of endogenous filamin B with ICAM-1. Importantly, clustering of ICAM-1 promotes the ICAM-1-filamin B interaction. To investigate the functional consequences of filamin B binding to ICAM-1, we used siRNA to reduce filamin B expression in ICAM-1-GFP expressing HeLa cells. We found that filamin B is required for the lateral mobility of ICAM-1 and for ICAM-1-induced transmigration of leukocytes. Reducing filamin B expression in primary human endothelial cells resulted in reduced recruitment of ICAM-1 to endothelial docking structures, reduced firm adhesion of the leukocytes to the endothelium and inhibition of transendothelial migration. In conclusion, this study identifies filamin B as a molecular linker that mediates ICAM-1-driven transendothelial migration.
INTRODUCTION

Endothelial cells are highly specialized to create a relatively impermeable barrier between the circulating blood and underlying tissue. The endothelium plays an important role in the migration of leukocytes across the vascular barrier, which is essential for host defence and is an important aspect of inflammatory diseases such as rheumatoid arthritis or atherosclerosis (Stemme and Hansson, 1994). Transendothelial migration involves initial tethering of activated leukocytes to the inflamed vessel wall, followed by the rolling of leukocytes over the endothelium (Springer, 1994; Vestweber, 2007). The leukocytes then firmly adhere, which is followed by spreading and migration across the endothelium. Firm adhesion between leukocytes and the endothelium is mediated by interactions between leukocyte integrins and endothelial immunoglobulin-like adhesion molecules (Ig-CAMs), such as the intercellular cell adhesion molecule-1 (ICAM-1) (Lyck et al., 2003; Reiss et al., 1998; Barreiro et al., 2002; Greenwood et al., 1995; Oppenheimer-Marks et al., 1991).

The resting endothelium expresses ICAM-1 at low levels. However, upon activation by inflammatory stimuli the expression of ICAM-1 is markedly increased, to promote integrin-mediated adhesion and transmigration of leukocytes (Roebuck and Finnegan, 1999). ICAM-1 is composed of five extracellular Ig-like repeats, a single transmembrane spanning part and a short intracellular domain consisting of twenty-eight amino acids. The cytoplasmic domain of ICAM-1 is implicated in the activation of signal transduction pathways and transendothelial migration (Greenwood et al., 2003; Lyck et al., 2003; van Buul et al., 2007b). Clustering of ICAM-1 initiates endothelial signalling which includes activation of small GTPases such as RhoA, Rac1 and RhoG (Sans et al., 2001; Thompson et al., 2002; Etienne-Manneville et al., 2000; Etienne et al., 1998; Greenwood et al., 2003; Lyck et al., 2003; van Buul et al., 2007a) and of Src-kinase (Durieu-Trautmann et al., 1994; Wang et al., 2003) and of p38 MAP kinase (Rauch et al., 2007). It is believed that these pathways contribute to the transient reduction of endothelial integrity and thereby facilitate efficient leukocyte transendothelial migration (TEM). However, the proximal signalling events induced by ICAM-1 are unknown. The short intracellular domain of ICAM-1 contains no identified signalling motifs but several proteins have been reported to interact or co-localize with ICAM-1, some of which have been implicated in actin crosslinking, such as α-actinin, ezrin and moesin (Romero et al., 2002; Carpen et al., 1992; Heiska et al., 1998; Barreiro et al., 2005).

The interaction of leukocytes with the endothelium induces the formation of large membrane protrusions known as endothelial docking structures or transmigratory cups (Barreiro et al., 2002; Carman et al., 2003). Integrin ligands such as ICAM-1 and VCAM-1 are recruited to these structures together with specific cytoskeletal and signalling molecules (Barreiro et al., 2002; van Buul et al., 2007b). The current hypothesis is that the assembly of these structures supports the formation of multi-molecular complexes facilitating endothelial signalling, important for the transmigration of leukocytes (Barreiro et al., 2002; Carman et al., 2003).
In a search for proteins that mediate Ig-CAM signalling in endothelial cells, we used biotinylated peptides encoding the intracellular domains of ICAM-1, followed by isolation and identification of bound proteins. Here, we show that ICAM-1 clustering induced the association between ICAM-1 and filamin, a protein implicated in actin-crosslinking (Cunningham et al., 1997; Stossel et al., 2001; Tseng et al., 2004; van der and Sonnenberg, 2001; Barreiro et al., 2002). Reducing filamin expression in primary human endothelial cells results in impaired ICAM-1 clustering and membrane dynamics and reduced adhesion and transendothelial migration of leukocytes. Our study uncovers filamin as an important component of the proximal signalling events downstream from ICAM-1 in endothelial cells.

RESULTS
Identification of proteins associating to the intracellular domain of ICAM-1
Although the intracellular domain of ICAM-1 is short (28 amino acids) and contains no identified signalling domains, it has been shown that the lack of the intracellular tail of ICAM-1 results in a decrease of leukocyte transmigration and a loss of formation of apical cups around adhered leukocytes (Greenwood et al., 2003; Lyck et al., 2003; Oh et al., 2007; van Buul et al., 2007a). These results indicate that the ICAM-1 intracellular tail initiates signalling pathways involved in TEM. To identify proteins that bind to the intracellular domain of ICAM-1 we performed pull-down assays with lysates of primary Human Umbilical Vein Endothelial Cells (HUVEC) using biotinylated peptides encoding the intracellular tail of ICAM-1. Following capture with streptavidin-coated beads, peptide-bound proteins were separated by SDS-PAGE and visualized by silver staining (Figure 1A). A peptide encoding the VCAM-1 intracellular domain was used as a specificity control. The most prominent protein band specifically interacting with the ICAM-1 peptide (migrating at an MW above 200 KDa) was analysed by mass spectrometry and identified as the cytoskeletal linker protein filamin B.

To confirm the interaction of filamin B with the intracellular domain of ICAM-1, peptide-binding proteins were separated by SDS-PAGE and filamin B was identified by Western blot. Figure 1B shows that filamin B specifically binds to the intracellular domain of ICAM-1 and not to the intracellular domain of VCAM-1 or to streptavidin beads. There are three filamin homologues expressed in human, filamin A and filamin B which are ubiquitously expressed, and a muscle-specific filamin C. Since filamin A and B share 70% sequence homology, we also tested the binding of filamin A to the ICAM-1 cytoplasmic domain. Like filamin B, filamin A also interacts with the cytoplasmic domain of ICAM-1, albeit less efficiently (Figure 1B). In addition to filamin B, we detected another protein of approximately 20 kDa, binding to ICAM-1 (Figure 1A). This protein was identified by Western Blotting as caveolin-1. Recently, caveolin-1 has been reported to play a key role in ICAM-1-mediated transendothelial migration.
Filamin B mediates ICAM-1-driven transmigration of T-lymphocytes (Millan et al., 2006). Moreover, caveolin-1 was previously reported to interact with filamin A and filamin B (Millan et al., 2006; Stahlhut and van Deurs, 2000). Thus, ICAM-1 may form a complex with filamin proteins and caveolin-1 to mediate leukocyte transmigration.

**Figure 1.** The intracellular domain of ICAM-1 interacts with filamin B and caveolin-1. (A) HUVEC lysates were incubated with beads, coated with biotinylated peptides encoding the intracellular domain of ICAM-1, of VCAM-1 or with empty beads (ctrl) and analysed by SDS-PAGE and silver staining. ICAM-1-interacting proteins were identified by mass spectrometry; upper arrowhead, filamin B; lower arrowhead caveolin-1. (B) The interaction of filamin A, filamin B and caveolin-1 with the intracellular domain of ICAM-1 was corroborated by SDS-PAGE followed by Western blot. Left panels show whole cell lysates (WCL). Right panels show analysis of pull-down (PD) experiments with empty beads (ctrl) or the C-terminal peptides (C-term) as indicated. (C) The interaction of filamin B domain 19-24 with ICAM-1 was tested by expressing an HA-tagged domain 19-24 of filamin B. Left panel shows expression of the construct in lysates. Right panel shows interaction of the HA-tagged filamin B (19-24) with the intracellular tail of ICAM-1 (PD: C-term). (D) GST-filamin B (19-24) or a splice variant of filamin B, lacking the amino acids 2082–2122 (GST-filamin B-var-GST) were isolated and incubated with the ICAM-1 or VCAM-1 C-terminal peptides; bound proteins were analysed by western blot.
Filamin is a 280 kDa protein comprising an N-terminal actin-binding domain and a rod domain composed of 24 homologous repeats. We tested which region in filamin is involved in ICAM-1 binding. Since most reported interactions involve the C-terminal region of filamin B, we transfected a construct encoding repeats 19-24 of filamin B in Cos7 cells and performed a pull-down experiment with the C-terminal peptide of ICAM-1. The results in figure 1C showed that filamin B (19-24) construct binds the ICAM-1 peptide (Figure 1C). A construct containing domains 19-24 of filamin A also interacts with the ICAM-1 peptide (data not shown). To address if this region of filamin B binds directly to the intracellular tail of ICAM-1, a GST-fusion protein encoding filamin B domains 19-24 was isolated and incubated with C-terminal peptides of either ICAM-1 or VCAM-1. Using streptavidin-beads, peptide-binding proteins were precipitated and blotted for GST. The results showed that the ICAM-1 C-terminal region directly interacts with filamin B (Figure 1D). The VCAM-1 peptide showed only low binding to GST-filamin B (19-24) domain. Interestingly, the peptides did not interact with a splice variant of filamin B, lacking the amino acids 2082–2122 that span repeats 19 and 20, filamin B var-1(19-24) (van der et al., 2002) (Figure 1D).

Endogenous filamin and caveolin-1 interact with endogenous ICAM-1.

To study the interaction between endogenous ICAM-1 and filamin B, TNF-α-stimulated primary HUVEC were used. Western blot and flow cytometry analysis confirmed that overnight stimulation with TNF-α induced an increase in ICAM-1 expression in HUVEC (Figure 2A). Primary HUVEC were grown to confluency on FN-coated dishes, stimulated with TNF-α overnight and incubated for 30 minutes with magnetic αICAM-1 antibody-coated beads to induce clustering of ICAM-1. The cells were lysed and the magnetic beads were extracted from the lysate using a magnetic particle concentrator pen. SDS-PAGE followed by Western blot analysis of beads-associated proteins demonstrated that endogenous ICAM-1 could be effectively isolated using this method (Figure 2B). Likewise, αVCAM-1 antibody-coated beads but not IgG could extract VCAM-1 protein (Figure 2B). A small amount of ICAM-1 was repeatedly found associated to VCAM-1.

Analysis of the binding of filamin B and caveolin-1 demonstrated the co-precipitation of these proteins with the αICAM-1-coated beads, whereas these proteins were not detected using αVCAM-1, αIgG isotype control or αMHC antibody-coated beads (Figure 2C and data not shown). Interestingly, addition of the αICAM-1 antibody-coated beads to an endothelial cell lysate did not result in the co-precipitation of detectable amounts of filamin B (Figure 2D), indicating that the interaction with endogenous filamin B is promoted upon clustering of ICAM-1.

Caveolin-1 binds indirectly to ICAM-1.

To determine whether filamin is required for the caveolin-1-ICAM-1 interaction, a pull-down assay using the ICAM-1 C-terminal peptide was performed with lysates of cells treated with siRNAs for filamin A, -B or caveolin-1. In cells with reduced caveolin-1
levels, the interaction of filamin A and B with the ICAM-1 cytoplasmic tail remained intact (Figure 3). Knockdown of filamin A reduced the binding of caveolin-1 to the ICAM-1 peptide, whereas reducing filamin B had little to no effect on the binding of caveolin-1 to the ICAM-1 C-terminus (Figure 3). These data suggest that the ICAM-1-caveolin-1 interaction is primarily mediated through filamin A. Although it has been reported that filamin B also binds caveolin-1 (Millan et al., 2006; Stahlhut and van

**Figure 2.** Filamin B interacts with endogenous ICAM-1. (A) ICAM-1 protein is up-regulated after TNF-α-treatment in primary human endothelial cells. Left panel shows Western blot analysis of ICAM-1 expression in HUVEC. TNF-α treatment is in hours. Actin is included as a loading control. Bar graph shows expression of ICAM-1, analysed by flow cytometry. MFI, mean fluorescence intensity. Data are representative for at least 3 independent experiments. (B) Magnetic beads coated with anti-ICAM-1 or -VCAM-1 antibodies or with control IgG were incubated for 30 min on a monolayer of TNF-α-stimulated HUVEC, followed by lysis. The beads were used for specific immunoprecipitation (IP) of the various indicated proteins. WCL: whole cell lysate. (C) Proteins were isolated from cell extracts as described under B and IPs were analysed for presence of filamin B or caveolin-1. Filamin B levels in the lysates are included as loading controls. (D) Anti-ICAM-1 antibody-coated magnetic beads were added to intact cells (‘pre-lysis’), or to endothelial cell lysates (‘post-lysis’). ICAM-1 associates with filamin B when clustered by anti-ICAM-1 antibody-coated magnetic beads (left lane). ICAM-1 antibody-coated magnetic beads added post-lysis bound ICAM-1, but did not efficiently co-precipitate filamin B (left lanes). Lower panel shows loading control for filamin B. WCL, whole-cell lysate. Data are representative for 3 independent experiments.
Figure 3. Caveolin-1 interacts with ICAM-1 primarily through filamin A. HeLa cells treated with siRNA for filamin A, filamin B, caveolin-1 (Cav1) or control (ctrl) were used in a pull-down assay using the biotinylated peptide encoding the intracellular domain of ICAM-1. Cell lysates and isolated proteins were analysed by western blot for caveolin-1, filamin A, filamin B and tubulin (protein loading control).

Deurs, 2000), we could not detect a significant contribution of filamin B to the ICAM1-caveolin-1 interaction.

Filamin co-localizes with ICAM-1 upon ICAM-1 clustering.

Because filamin B was initially identified as the major isoform that bound the ICAM-1 C-terminus (Figure 1A), we focused in subsequent experiments on filamin B. The distribution of ICAM-1 and filamin B was analysed by immunocytochemistry in combination with confocal microscopy. Filamin B staining showed minimal co-localisation with ICAM-1 (Figure 4A). However, upon crosslinking of ICAM-1 using soluble αICAM-1 antibodies, ICAM-1 is redistributed to cell borders and to punctated structures on the cell body, in good agreement with findings by others (Wojciak-Stothard et al., 1999). Leukocyte adhesion to the endothelial surface results in the recruitment of ICAM-1 to sites of adhesion (Stahlhut and van Deurs, 2000; Carman et al., 2003; Barreiro et al., 2005; van Buul et al., 2007a; Oh et al., 2007). To mimic leukocyte binding via ICAM-1, we incubated primary HUVEC with αICAM-1-antibody coated beads and found that filamin B was recruited to these beads, co-localizing with ICAM-1 at sites of adhesion (Figure 4B). Similar results were obtained when differentiated HL-60 cells were used (S.Figure 1). To control for the specificity of the staining, we used αVCAM-1 or αMHC antibody-coated beads that also adhered to activated endothelial cells. The results showed that these beads recruited filamin B only to a limited extent (Figure 4B and data not shown), which is in agreement with the biochemical data in figure 2D. This indicates that efficient filamin B recruitment depends on ICAM-1 clustering.
ICAM-1-GFP expression in HeLa cells

To study the functional relevance of the interaction of filamin B with ICAM-1, we used HeLa cells that stably expressed ICAM-1-GFP. HeLa cells express only very low levels of endogenous ICAM-1, compared to TNF-α-treated endothelial cells (data not shown). Western blot analyses showed that the transfected cells express the ICAM-1-GFP protein (Figure 5A) and flow cytometry analysis indicated that approximately 90% of the HeLa cells stably expressed ICAM-1-GFP (Figure 5B). Confocal laser scanning microscopy revealed that ICAM-1-GFP was localized to apical microvilli (see also Figure 6B) and recruited around αICAM-1-antibody coated beads (Figure 5C), showing that the ICAM-1-GFP fusion protein is functional. The recruitment of ICAM-1-GFP was visualized in more detail using Z-stack imaging (Figure 5D, Video 1). This analysis shows that ICAM-GFP is clearly recruited in the structure surrounding the adherent bead that can protrude up to 5 micron from the plane of the membrane.

Filamin regulates ICAM-1 membrane dynamics.

To explore the role of the filamin B-ICAM-1 interaction, we used siRNA to reduce filamin B expression in ICAM-1-GFP expressing HeLa cells. Western blot analysis showed that filamin B siRNA efficiently reduced the levels of endogenous filamin B in these cells (Figure 6A). We initially analysed the role of filamin B in ICAM-1 membrane dynamics. To this end we used FRAP (Fluorescent Recovery After Photo-bleaching) analysis.
FRAP analysis of the membrane-associated GFP-CAAX protein revealed no difference in the fluorescence recovery between filamin B-siRNA treated cells and control cells (Figure 6A). FRAP analysis of ICAM-1-GFP showed that reduced expression of filamin B resulted in a decreased fluorescence recovery of ICAM-1-GFP (Figure 6B, Videos 2 and 3). This data suggest that filamin B regulates ICAM-1-GFP mobility in the plasma membrane. Note from the videos that the distribution of ICAM-1 GFP in the filamin
B siRNA treated cells is much more diffuse throughout the plasma membrane, when compared to the controls, which show more discrete apical structures in which ICAM-1-GFP is concentrated.

Since filamin B is an adaptor protein that is linked to the sub-cortical actin cytoskeleton (Matsudaira, 1994), filamin may regulate cytoskeletal dynamics and thereby directly or indirectly, control the mobility of ICAM-1. Detailed analysis of the data showed that reduction of filamin B expression significantly reduced the mobile fraction of ICAM-1 but not of the GFP-CAAX (Figure 6C). Interestingly, by calculating the slope of the curves, depicted in figure 6A and B, the data indicated that filamin B knockdown did not affect the speed of recovery (Figure 6D). Finally, we

![Graphs and images showing FRAP analysis](image)

**Figure 6. Filamin mediates lateral membrane mobility of ICAM-1.** FRAP analysis in combination with confocal imaging was used to study membrane dynamics of GFP-CAAX as a control (A) and ICAM-1-GFP (B) in HeLa cells transfected with control (grey line) or filamin B (dark line) siRNA. Western blot in A shows expression analysis of filamin B. Tubulin is included as a loading control. Dashed lines indicate pre-bleaching intensity set at 100%. (A) The fluorescence recovery of GFP-CAAX was not affected by reduced expression of filamin B. Graph is representative of 4 independent experiments. (B) Images show ICAM-GFP distribution in control siRNA or filamin B siRNA treated cells. Bar, 20 μm. In control siRNA treated cells, ICAM-1-GFP recovered to approximately 70% within 2.5 minutes (grey lines), whereas filamin B-deficient cells showed recovery to maximal 40% (dark lines). Graph is representative of 4 independent experiments. (C) Mobile fractions of both experiments are calculated and show that filamin B siRNA significantly reduces the mobile fraction of ICAM-1-GFP but not GFP-CAAX. Data are mean±SEM of 4 independent experiments; *p<0.01. (D) Measurement of the slope (K) of the recovery depicted in A and B shows that reduction of filamin B did not affect the speed of motility of ICAM-1-GFP or of GFP-CAAX in the plasma membrane. Data are mean±SEM of 4 independent experiments.
did not observe major changes in cytoskeletal architecture following treatment of the endothelial cells with filamin B siRNA. Together, these data suggest that filamin B links ICAM-1 to the actin cytoskeleton and that filamin B promotes the extent rather than the speed of ICAM-1 lateral mobility within the plasma membrane, without directly affecting cytoskeletal dynamics.

Filamin mediates efficient recruitment of ICAM-1 upon clustering.

Next, we tested whether filamin B is required for ICAM-1 clustering. Recruitment of ICAM-1 to αICAM-1 antibody-coated beads was analysed by time-lapse confocal laser scanning microscopy using HeLa cells stably expressing ICAM-1-GFP in the absence or presence of filamin B. For the immunofluorescence experiments, siGLO was transfected together with filamin B siRNA, to certify that the cells used for imaging were indeed transfected with siRNA (data not shown). The antibody-coated beads induced recruitment of ICAM-1-GFP in control siRNA treated cells within approximately 8 minutes (Figure 7A, Video 4). GFP-CAAX, used as a control, was not significantly recruited to ICAM-1 antibody-coated beads (data not shown). Reducing filamin B expression with siRNA revealed that ICAM-1 was eventually recruited to adherent beads, albeit with a delay of 10-15 minutes (Video 5). To complement the analysis in figure 7A, the contribution of filamin for the formation of cup structures was quantified using cells treated with control or filamin B siRNA. In the absence of filamin B, a significant reduction in the number of cup structures formed was found (figure

![Image](image_url)

**Figure 7. Filamin B mediates recruitment of ICAM-1.** (A) αICAM-1 antibody-coated beads were incubated with ICAM-1-GFP expressing Hela cells and the recruitment of ICAM-1-GFP to the beads was recorded for 20 min. using time-lapse confocal imaging. Time in minutes is shown above the images. Cells were treated with control siRNA or filamin B siRNA, as indicated on the left. Still images in panel A show ICAM-1-GFP recruitment (green) around ICAM-1 antibody-coated beads in siRNA control cells after approximately 4 to 8 minutes (open arrowheads upper panels), whereas ICAM-1-GFP recruitment occurs not before 20 minutes in cells with reduced filamin B expression (lower images). Bar, 10 μm. Lower panels show line-scan of the fluorescent intensity of ICAM-1-GFP surrounding the bead, indicated by the white dashed bar. (B) Quantification of the number of cup structures, formed around adherent beads on cells treated or not (ctrl) with siRNA to filamin B. *, p<0.001.
Filamin B mediates ICAM-1-driven transmigration. Together, these data indicate that filamin B controls lateral motility of ICAM-1 and thereby mediates its recruitment to sites of ICAM-1 engagement.

**Filamin is required for ICAM-1-mediated transendothelial migration.**

To test if filamin plays a role in ICAM-1-mediated transmigration of leukocytes, HeLa ICAM-1-GFP cells were cultured to confluency on Transwell filters and treated with filamin B or control siRNA. Adhesion of αICAM-1-antibody coated beads (Figure 8A) was decreased in filamin B-deficient cells. Next, differentiated HL60 cells were allowed to migrate across ICAM-1-GFP expressing HeLa cells towards the chemokine SDF-1 in a Transwell-based chemotaxis assay. The results showed that introducing ICAM-1-GFP into HeLa cells was sufficient to stimulate leukocyte chemotaxis toward SDF-1 (Figure 8B). Reducing filamin B protein levels resulted in an inhibition of ICAM-1-induced migration of leukocytes, close to levels of SDF-1-induced migration across HeLa cells that did not express ICAM1-GFP (Figure 8B). To study the role of filamin B on the function of endogenous ICAM-1, filamin B expression was reduced in primary endothelial cells using siRNA. Subsequently, transmigration of cells across the endothelium was recorded under flow, and quantified based on their transition from phase-bright to phase-dim (Figure 8C). These experiments showed that transendothelial migration of leukocytes in the absence of filamin B was reduced (Figure 8D). Note that also the initial adhesion of the leukocytes to ICAM-1 was affected in filamin B-deficient primary endothelial cells (Figure 8D). These data show that filamin B plays an important, non-redundant role in the adhesion and subsequent transmigration of leukocytes across the endothelium by regulating ICAM-1 recruitment and function.

**DISCUSSION**

Transendothelial migration (TEM) of leukocytes is the result of a dynamic interplay between activated leukocytes and endothelial cells. Leukocytes bind through integrins to their ligands, such as ICAM-1, expressed on endothelial cells, which is crucial for efficient TEM of various types of leukocyte (Figure 8 and (Celli et al., 2006; Sans et al., 2001)) The binding to ICAM-1 results in ICAM-1 clustering and subsequent induction of intracellular signalling, facilitating the passage of leukocytes (van Buul et al., 2007b). ICAM-1 activates a wide range of signalling pathways, including protein tyrosine phosphorylation, Rho-like GTPase activation and modulation of cytoskeletal dynamics. Most of these signalling events apparently also feed back to ICAM-1, as these have all been implicated in ICAM-1 clustering, leukocyte adhesion and TEM.

The ICAM-1-filamin interaction

The ICAM-1 intracellular domain is critical for ICAM-1 function and signalling and deleting this region impairs transmigration of leukocytes (Celli et al., 2006; Sans et al., 2001; Greenwood et al., 2003; Lyck et al., 2003). However, the fact that ICAM-1 intracellular domain is very small, lacking any established signalling motifs, raises
Figure 8. Filamin B regulates ICAM-1-mediated adhesion and transmigration under static and flow conditions. (A) Reduced filamin B levels reduced the adhesion of αICAM-1 antibody-coated beads to ICAM-1-GFP expressing HeLa cells. Beads were counted per field of view, 20 fields per experiment were counted and three independent experiments have been carried out. HeLa cells were either not treated (NT), or transfected with ICAM-1-GFP and treated with control siRNA (Ctrl) or filamin B siRNA as indicated. Data are mean +/- SEM of a representative experiment performed in quadruplicate. *p<0.05 (B) Differentiated HL60 cells were allowed to migrate across a HeLa ICAM-1-GFP monolayer in a Transwell system to 50ng/mL SDF-1. Percentage migration was determined as described in Materials and Methods. HeLa cells were either not treated (NT), or transfected with ICAM-1-GFP and treated with control siRNA (Ctrl) or filamin B siRNA as indicated. Data are mean +/- SEM of a representative experiment performed in quadruplicate. *p<0.05 (C) Differentiated HL60 cells were perfused over monolayers of TNF-α-treated primary human endothelial cells. Migration was visualized by adhesive cells crossing the monolayer thereby changing from a bright to a dim appearance (arrowheads). (D) Differentiated HL60 cells were flown over monolayers of TNF-α-treated primary human endothelial cells. Left panel shows quantification of leukocyte migration across endothelial monolayers transfected with filamin B siRNA (si-filamin B), compared to migration across siRNA control treated endothelial cells (siCtrl). Right panel shows quantification of adhesion of HL60 cells to endothelial cells treated with siRNA to filamin B. Data represent averages of duplicates from one out of two independent experiments.
questions about the nature of protein-protein interactions that govern ICAM-1-driven signalling. In this study we show that the intracellular domain of ICAM-1 binds the actin-crosslinking proteins filamin A and, in particular, filamin B. This interaction is specific, in that the intracellular domain of VCAM-1 does not bind to filamin B. Moreover, filamin B binding to ICAM-1 is stimulated by antibody-mediated clustering, suggesting that the ICAM-1-filamin B association is induced following the binding of leukocytes to endothelial cells. The ICAM-1-filamin complex also comprises caveolin-1. Our data suggest that caveolin-1 associates to ICAM-1 through filamin, in particular filamin A. Whether indeed two functionally distinct complexes, comprising filamin B and filamin A-caveolin-1, form upon ICAM-1 clustering is as yet unknown and will be a topic of future studies.

Millán and colleagues reported that ICAM-1 travels with the leukocyte to the baso-lateral site of the endothelial cells (Millan et al., 2006). Leukocytes that use the transcellular route, i.e. through the endothelial cells, use caveolae as a starting point. This pathway depends on caveolin protein, since reduced expression of caveolin results in an inhibition of transcellular migration. Our data link caveolin to ICAM-1 through filamin, in line with a recent report which showed that caveolin and filamin interact (Stahlhut and van Deurs, 2000). In addition, the fact that filamin B binding to ICAM-1 is increased upon ICAM-1 clustering is in agreement with the data by Millán and co-workers, who showed that the F-actin rings, formed around adhered leukocytes, are most likely mediated by filamin (Millan et al., 2006). Finally, our findings are also similar to what was described for the CD4-filamin A interaction, which is increased upon CD4 ligation (Jimenez-Baranda et al., 2007).

Recently, it was published that the intracellular tail of ICAM-5 binds to alpha-actinin as well as to filamin (Nyman-Huttunen et al., 2006). However, by mapping the binding sites, it appears that alpha-actinin binds to the positively charged amino-acid region in ICAM-5, similar as is shown for its binding to ICAM-1 (Celli et al., 2006), but filamin did not bind this region, and may therefore bind to a more membrane-distal sequence in the ICAM-1 C-terminus. As for filamin B, our results show that the repeats 19-24 of filamin B, in particular repeats 19 and 20, are involved in the binding to the intracellular tail of ICAM-1. This conclusion is based on the pull-down assays using the GST-filamin B-variant construct, which lacks amino acids 2082–2122 and that appears to bind more strongly than the wild-type filamin B to different integrin beta subunits (van der et al., 2002). Our experiments show that the intracellular tail of ICAM-1 did not directly associate with the filamin B-variant construct, but did associate with the 19-24 repeats of filamin B wild type.

Thus, our data show that filamin B associates, as α-actinin and ERM proteins, with the intracellular domain of ICAM-1. Whether these membrane-proximal complexes comprising ICAM-1 form in parallel or in sequence or perhaps even at different positions in the plasma membrane following ICAM-1 clustering is presently unclear. Importantly, filamin B appears to serve a critical, non-redundant role following ICAM-1
clustering. This can be concluded from our findings that the ICAM-1 recruitment around anti-ICAM-1-coated beads, as well as the adhesion and transendothelial migration of leukocytes was reduced in primary endothelial cells expressing reduced levels of filamin B, even though these cells express filamin A.

Filamin in ICAM-1 mobility and signaling
Clustering of ICAM-1 results in an increase in stress fiber formation and transient activation of the small GTPase RhoA (Thompson et al., 2002), indicating that ICAM-1 signals toward the actin cytoskeleton. Filamins are excellent candidates in mediating this signalling as these proteins act as actin-binding scaffolds, interacting with cell-surface receptors such as integrins as well as signalling molecules such as caveolin-1, PKC and small GTPases (Stossel et al., 2001; Feng and Walsh, 2004). Preliminary findings showed that ICAM-1 crosslinking reduces endothelial cell-cell contact in a filamin B-dependent fashion (data not shown). The signalling function of filamin may well play a role in this pathway, as we and others have previously shown that various proteins such as small GTPases and protein kinases play a key role in the modulation of cell-cell contact through IgCAM-driven signalling (van Buul et al., 2007a). Intriguingly, Our FRAP experiments showed that siRNA-mediated knockdown of filamin B reduces the mobile fraction of ICAM-1 in the membrane. This is in line with the reduced recruitment of ICAM-1 to antibody-coated beads in the absence of filamin B and suggests that filamin B-controlled cytoskeletal dynamics and/or its binding to ICAM-1, promotes ICAM-1 translocation in the plane of the membrane. Thus, ICAM-1 mobility is apparently driven from within the cell, depends on a proper interaction with the cortical actin cytoskeleton network and is enhanced after crosslinking, which promotes filamin B binding. This notion is in agreement with data by Yang et al. (Yang et al., 2006), who showed that src-mediated phosphorylation of the actin crosslinker cortactin regulates ICAM-1 mobility. Similar as for filamin B, reduced cortactin expression impaired clustering of ICAM-1.

Recent data indicate that F-actin/filamin networks are stiffer and less dynamic compared to F-actin/α-actinin networks (Tseng et al., 2004). Our data show that clustering of ICAM-1 promotes the association of filamin to ICAM-1 and that reduced filamin levels decreased the adhesion of leukocytes to ICAM-1 (Figure 8D). This suggests that a more rigid actin network, regulated by filamin, is required for optimal ICAM-1 function, i.e. the binding of leukocytes.

Whereas binding to actin-crosslinking proteins thus promotes ICAM-1 recruitment, blocking actin polymerization by cytochalasin D also increases the mobile fraction of ICAM-1 upon clustering (JDvB, unpublished results). Similarly, under these conditions, the formation of apical cup-structures is impaired, although the adhesion of leukocytes remains unaltered (Carman et al., 2003). An emerging concept is therefore that the ICAM-1-cytoskeleton connection represents a bidirectional signalling module that coordinates the mobility of IgCAMs such as ICAM-1, which, upon clustering, signal toward actin and mediates increased actin polymerization. This may likely represent
a positive feedback loop that serves not only to recruit ICAM-1, but also to secure a coordinated link to a more rigid cortical actin network, which is required for proper adhesion and efficient migration of leukocytes over the endothelial apical membrane. This molecular interplay may in fact be quite similar to the situation for integrin-mediated adhesion and consequent formation of focal adhesions, which promote as well as depend upon the interaction with the actin cytoskeleton.

The above results put filamin B forward as a crucial regulator for ICAM-1 function in the process of transendothelial migration of leukocytes. Filamin B not only binds to and regulates ICAM-1 lateral mobility, but also, through its crosslinking activity, promotes formation of a rigid actin network that supports ICAM-1-mediated leukocyte extravasation, an event that is critical in immune surveillance and inflammation.
MATERIALS AND METHODS

Cell culture and transfection
HEK-293, Cos7, HL60 and HeLa were maintained in IMDM (Biowhittaker, Verviers Belgium) containing 10% heat inactivated Fetal Calf Serum (FCS; Life Technologies, Breda The Netherlands), 300 μg/ml glutamine, 100 units/ml penicillin and streptomycin at 37°C and 5% CO₂. Primary HUVECs were purchased from Cambrex (Baltimore M.D.) and cultured during regular passaging and for experiments following FN (10μg/ml) coating of the (Sigma) tissue culture flasks (Invitrogen, Breda, The Netherlands) or glass slides in EGM2 containing singlequots (Lonza Baltimore M.D.). Endothelial cells were cultured until passage 9. For siRNA transfection experiments early passages (≤5) were used.

siRNA transfections
For siRNA-mediated down regulation of Caveolin-1, filamin A and filamin B the following sequences were used: Caveolin-1: 5’-AAUCUAUCAGGAAGCUC-3’; filamin A: 5’-CACAGAAUUGACCAAGAUAGUAU-3’; filamin B: 5’-GCCCAUUACCGUGAAGAU-3’.
Oligos were purchased from Eurogentec (Liege, Belgium); control siRNA was used from Dharmacon (Perbio, Etten-Leur, The Netherlands). An expression construct for ICAM-1-GFP was kindly provided by Dr. F. Sanchez-Madrid (University of Madrid, Madrid, Spain), HA-tagged filamin B and filamin A repeats 19-24 as well as the GST-filamin B 19-24 and the GST-filamin B-var 19-24 were a kind gift from Dr. A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, The Netherlands). Double strand siRNA oligos were transfected in HUVEC using Interferin transfection reagent and transfection medium (Tebu-Bio, Heerhugowaard, The Netherlands) according to the manufacturer’s protocol. Oligofectamine (Invitrogen) was used to transfect siRNA oligos and expression constructs in HELA cells. Cells were transfected when ~70% confluent. 0.300 μMol of siRNA was diluted in 250 μl Opti-Mem and left for 5 minutes at room temperature. 15 μl Oligofectamine was mixed with 60 μl Opti-Mem and incubated for 5 minutes at room temperature. Next, the two mixtures were pooled and incubated for 20 minutes at room temperature. The mixture was then added to the cells for 6 hours and replaced with fresh culture medium. Both HeLa cells and HUVEC were used for experiments 48 hours after transfection.

Antibodies
The monoclonal antibody (mAb) to ICAM-1 and anti-ICAM-1-FITC were purchased from R&D systems (Minneapolis, USA); the anti-VCAM-1 mAb was from Beckman Coulter (Marseille, France); the goat polyclonal Ab to VCAM-1 (C-19) was purchased from Tebu-Bio. filamin A mAb was purchased from Serotec (Oxford UK); polyclonal filamin B Ab was from Millipore (Amsterdam, The Netherlands); the polyclonal caveolin-1 Ab was from BD Transduction Laboratories (Breda, The Netherlands); mAb anti-HA (12CAS) was from Boehringer (Almere, The Netherlands); isotype control IgG was from Sanquin, Amsterdam, The Netherlands; Alexa-488-labelled chicken-anti-mouse, Alexa-488 chicken-anti-rabbit; Alexa-594 chicken-anti-mouse; Alexa-594 chicken-anti-rabbit and Alexa-647 chicken-anti-goat were purchased from Invitrogen (Leiden, The Netherlands). For F-actin staining Bodipy-650/665-phalloidin and Texas Red-phalloidin were used (Invitrogen).

Coating of beads
Magnetic goat anti-mouse IgG-coated Dynabeads (Invitrogen) were coated with ICAM-1 mAb, VCAM-1 mAb or IgG isotype control according to the manufacturers’ protocol. In short; beads were washed with ice cold PBS (+0.1% BSA; 2 mM EDTA) and incubated with 1 μg antibody per 1 x 10⁷ beads for 45 min. at 4°C under constant head over head rotation. The beads were subsequently washed twice to remove any unbound antibody. Polystyrene 10 and 3 μm beads (Polysciences Inc., Warrington, PA, USA) were coated with antibody according to the manufacturers’ instructions. Beads were washed in PBS and incubated overnight with glutaraldehyde at room temperature under constant rotation. Beads were then washed three times with PBS and incubated with 1 μg antibody per 2x10⁶ beads for 4-5 hours. After washing the beads they were incubated in 0.5 M ethanolamine in PBS for 30 minutes and subsequently washed and blocked with 10 mg/ml BSA in PBS for 30 min.
SDS-PAGE, Western Blotting and silver staining
SDS-PAGE samples were analyzed on 7.5, 10 or 12.5% polyacrylamide gels depending on the size of the proteins of interest and transferred onto PVDF membrane (Biorad). Following blocking in 5% low fat milk in TBST (Tris-buffered saline Tween-20) the blots were incubated with the primary antibody overnight at 4°C, washed 3 times for 10 min. in TBST and subsequently incubated with HRP-coupled secondary antibodies (dilution: 1:7000) in TBST for 1.5 hours at room temperature followed by 3 times washing with TBST for 20 min. each and development of the blot by ECL (GE Healthcare, Hoevelaken, The Netherlands). For silverstaining, and subsequent mass spectrometry, pre-cast gradient 4-12% Bis-Tris gel (Invitrogen) was used according to manufacturers’ instructions.

In-gel digestion and mass spectrometry
Protein bands were excised from SDS-PAGE gels, reduced, alkylated and in-gel digested using trypsin (modified, sequencing grade, Promega) as previously described (Steen et al., 2002). After digestion, peptides were collected using two rounds of extraction with 20 μl of 0.1 % TFA and stored at –20 °C prior to analysis by mass spectrometry. For LC-MS analysis, samples were injected onto a nano-LC system (Ultimate, Dionex, Amsterdam, the Netherlands) equipped with a peptide trap column (Pepmap 100, 0.3 i.d. x 1 mm) and an analytical column (Pepmap 100 , 0.075 i.d x150 mm, Dionex, Amsterdam, the Netherlands). The mobile phases consisted of (A) 0.04 % formic acid/0.4 % acetonitrile and (B) 0.04% formic acid/90% acetonitrile. A 45 min linear gradient from 0 to 60% B was applied at a flow rate of 0.2 μl/min. The outlet of the LC system was coupled to an HCT ion-trap mass spectrometer (Bruker Daltonics, Bremen) using a nano-electrospray ionisation source. Eluting peptides were analyzed in the data dependent MS/MS mode over a 400-1600 m/z range. Mass spectra were evaluated using the DataAnalysis 3.1 software package (Bruker Daltonics, Bremen, Germany). MS/MS spectra were searched against the human IPI database using the Mascot search algorithm (Matrix Science, London, UK), allowing mass tolerances of 1.5 Da for MS and 0.5 Da for MS/MS and one missed cleavage site. Carbamidomethylcysteine was taken as a fixed modification and oxidation of methionine as a variable modification.

Pull-down assay and immunoprecipitation
A synthetic, biotinylated peptide encoding the intracellular domain of human ICAM-1, VCAM-1 or empty beads were used in pull-down assays as previously described (ten Klooster et al., 2006). The following sequences were synthesized from N- to the C-terminus: ICAM-1: NH2-RQRKIKKYRLQQAQKTPPMKNTQPFP-COOH; VCAM-1: NH2-IIYFARKANMKGYSLVEAQKSKV-COOH. A confluent HUVEC monolayer in a 100 mm Petri-dish was washed with cold PBS (+ 1mM CaCl2; 0.5 mM MgCl2) and lysed in cold NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM MgCl2, 10% glycerol (v/v) and 1% Nonidet P40 (v/v)) supplemented with protease inhibitors (complete mini EDTA, Roche, Almere, The Netherlands) for 5 minutes. After lysis, cell debris was removed by centrifugation (14000 rpm, 5 min at 4°C). Supernatant was incubated with 5 μg biotinylated peptide comprising the intracellular tail of ICAM-1 or indicated controls and 30 μl (concentration of 2.4 mg/ml packed gel) streptavidine beads for 2-3 hours under constant head over head rotation at 4°C. Beads were subsequently washed five times with NP40 lysis buffer and resuspended in SDS-PAGE sample buffer. Alternatively, antibody-coated magnetic beads were resuspended in EGM2 and incubated on a HUVEC monolayer at a concentration of approximately 4-8 beads per cell for 30 min. unless otherwise indicated. For subsequent immuno-precipitation, cells were gently washed in cold PBS (supplemented with 1 mM CaCl2; 0.5 mM MgCl2) to remove unbound beads and lysed in cold RIPA buffer (1% Nonidet P40 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 50 mM Tris pH 7.4, 1 % deoxycholic acid (DOC), and 0.1% SDS Beads were extracted from cell lysates using a magnetic pen (PickPen 1M, BioNobile), and subsequently washed five times in a NP40-based lysis buffer (1% Nonidet P40 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 50 mM Tris pH 7.4) to reduce coagulation of the beads 15 u DNAse 1 (Fermentas, Germany) was added to the lysis buffer and in the first washing step. Finally, beads were resuspended in 30 μl SDS sample buffer for SDS-PAGE followed by Western blot analysis for ICAM-1. Additionally, a pull-down assay was
performed using GST- filamin B 19-24 and GST- filamin Bvar 19-24-GST recombinant protein as described by van der Flier et al. (van der Flier et al., 2002). GST proteins were purified from BL21 bacteria as described (41) and isolated from GST beads using PreScission Protease according to manufacturers’ protocol. Supernatant containing the filamin-GST proteins was used in a pull-down experiment as described above.

Transmigration and adhesion assay

Transmigration was analysed using Transwell permeable supports from Costar (d=6.5 mm; 5 μm pore size). A monolayer of HeLa ICAM-1GFP expressing cells were cultured on the filter and transfected with siRNA for filamin B or with control siRNA (Dharmacon). The cells were seeded at 2 x 10^5 cells per well 24 hours after transfection and subsequently cultured for an additional 24 hours. The monolayer was then washed once with migration medium (IMDM 2% FCS w/o antibiotics) and 1 x 10^5 differentiated HL60 cells (4 days 1.3% DMSO supplemented to culture medium) were transferred to the upper compartment and cells were allowed to migrate for 2 hours towards the lower chamber in a chemotactic gradient with SDF-1. The number of migrated HL60 cells is quantified by flow cytometry using count beads according to van Hennik et al. (2003). Following the migration assay, the monolayer was fixed, permeabilized, stained with Texas-RED phalloidin to visualize F-actin and analysed by microscopy to ensure that the monolayer was confluent.

Transmigration under flow – Primary HUVECs were cultured to confluency in EGM2 (Cambrex) on FN-coated glass covers transected with either control or filamin B siRNA according to manufactures protocol using siRNA transfection reagent (Santa Cruz CA ). Cells were treated with 10ng/mL TNF-α overnight. Next, cells were mounted onto the microscope stage using a POC-mini chamber system (LaCon, Staig, Germany) and connected to a perfusion pump. Using physiological flow conditions (5 dyns/cm²), 1*10⁶ differentiated HL60 cells per mL were perfused over the endothelial cells, followed by another 20 minutes of fluid-flow. Transmigration was characterized by adhesive cells crossing the monolayer during this time-frame, changing from a bright to a dim appearance (See Figure 8C). Migrated cells were quantified by counting per field-of-view. From one experiment, five fields were analyzed.

Adhesion of anti-ICAM-1 antibody-coated beads was analysed in a 24-wells plate containing FN-coated glass coverslips. HeLa-expressing ICAM-1-GFP cells, transfected with control or filamin B siRNA were cultured on the coverslips to confluency. Cells were left on ice for 3 min. and equal amounts of anti-ICAM-1-coated beads (10 μm diameter) were added. Cells were left on ice for an additional 3 min. to allow sedimentation of the beads onto the monolayer. Subsequently the beads were incubated at 37 °C for 5 min. and transferred onto ice again. Next, the cells were washed twice with ice-cold PBS and subsequently fixed with 4% formaldehyde in PBS. Confocal microscopy was used to make overviews at fixed positions in the coverslips. Subsequently the total number of beads were counted using Zeiss LSM Image software.

Confocal laser scanning microscopy

For immuno-fluorescence, HUVECs or HeLa ICAM-1-GFP cells were grown on FN-coated 14 mm or 30 mm coverslips. After treatment, cells were washed in cold PBSA (PBS; 0.1% BSA) and fixed in 4% formaldehyde in PBS for 10 min. After fixation, cells were permeabilized in PBS-T (PBS + 0.1% Triton X100) for 10 min. followed by a blocking step in PBS supplemented with 2% BSA. Cells were incubated with primary and secondary antibodies and after each step, washed 3 times in PBSA. Coverslips were mounted with vectashield (Vector laboratories Inc., Peterborough UK) or mowiol 4-88 reagent (Calbiochem Omnilabo, Breda, The Netherlands) on microscope slides. For life-cell imaging, HeLa ICAM-1-GFP, or controls were seeded on 30 mm coverslips and transfected with filamin B siRNA and siGlow (Dharmacon) according to the manufacturers’ instructions. Coverslips were transferred to the POCmini chamber system and mounted onto a heating block connected to a confocal microscope (Zeiss LSM510). FRAP (fluorescent recovery after photobleaching) experiments were performed using 30 iterations with 488 nm laser illumination, at maximum power (25 mWatt). Fluorescence recovery was measured by time-lapse imaging. Image analysis was performed with LSM 510 software (Carl Zeiss MicroImaging, Inc). Graphpad Prism 4 was used for statistical analysis and non linear regression. For curve fitting
a one exponential association was used (equation; Y=Ymax (1-exp(-K*X)) which starts at zero and ascends to Ymax with a rate constant K in which Ymax represents the mobile fraction and K represents the time characteristics of the curve.

ACKNOWLEDGMENTS
The authors would like to thank Dr. F. Sanchez-Madrid for providing the ICAM-1-GFP construct. We thank the members of the department of Molecular Cell Biology for helpful discussions. This work was supported by the Dutch Heart Foundation (grant nr. 2003B012); JDvB was supported by the Dutch Heart Foundation (grant no. 2005T039) and NWO Veni grant 916.76.053. JvR was supported by the Academic Medical Center, Amsterdam, the Netherlands. PLH is a fellow of the Landsteiner Foundation for Blood Transfusion Research (grant no. 0112).
REFERENCES


Figure 1. Distribution of ICAM-1 and filamin B at sites of leukocyte-endothelium interaction. (A) Endothelial cells were cultured and grown to confluence on FN-coated glass cover slips and differentiated HL60 cells were allowed to adhere to the endothelium for 30 minutes. Next, cells were fixed, permeabilized and stained for ICAM-1 (green, b,d), filamin B (red, b,d) and F-actin (white, a,c). Images were taken from the apical (a,b) and the baso-lateral focal plane (c,d) to visualize the adherent leukocyte (Asterisk). Bar, 10 μm. (B) To visualize co-localization between ICAM-1 and Filamin B, co-localization software (Zeiss) was used to analyze the apical images of the adherent HL60 cell. Pixels with above-threshold levels of both ICAM1 and Filamin B are represented in white (lower panel, arrowheads).
SUPPLEMENTAL VIDEOS

Video 1. Projection from a Z-stack shows recruitment of ICAM1-GFP into a cup structure, formed around an adherent anti-ICAM-1 coated bead on a Hela cell. Bead size is 10 micron.

Video 2. FRAP analysis of ICAM-1-GFP in Hela cells, transfected with control siRNA. Box indicates area used for calculation of recovery. Video is 10 frames/second. Time span of the video is 300 sec (100 x real time).

Video 3. FRAP analysis of ICAM-1-GFP in Hela cells, transfected with filamin B. Box indicates area used for calculation of recovery. Video is 10 frames/second. Time span of the video is 300 sec (100 x real time).

Video 4. Movie shows the recruitment of ICAM-1-GFP to beads, coated with anti-ICAM-1 antibodies, in Hela cells, transfected with control siRNA. Video is 10 frames/second. Time span of the video is 23 min (280 x real time).

Video 5. Movie shows the lack of recruitment of ICAM-1-GFP to beads, coated with anti-ICAM-1 antibodies, in Hela cells transfected with siRNA to filamin B. Video is 10 frames/second. Time span of the video is 23 min (280 x real time).