

Neisseria meningitidis infection of human endothelial cells interferes with leukocyte transmigration by preventing the formation of endothelial docking structures

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N*Neisseria meningitidis* elicits the formation of membrane protrusions on vascular endothelial cells, enabling its internalization and transcytosis. We provide evidence that this process interferes with the transendothelial migration of leukocytes. Bacteria adhering to endothelial cells actively recruit ezrin, moesin, and ezrin binding adhesion molecules. These molecules no longer accumulate at sites of leukocyte–endothelial contact, preventing the formation of the endothelial docking structures required for proper leukocyte diapedesis. Overexpression of exogenous ezrin or moesin is sufficient to

rescue the formation of docking structures on and leukocyte migration through infected endothelial monolayers. Inversely, expression of the dominant-negative NH₂-terminal domain of ezrin markedly inhibits the formation of docking structures and leukocyte diapedesis through non-infected monolayers. Ezrin and moesin thus appear as pivotal endothelial proteins required for leukocyte diapedesis that are titrated away by *N. meningitidis*. These results highlight a novel strategy developed by a bacterial pathogen to hamper the host inflammatory response by interfering with leukocyte–endothelial cell interaction.

Introduction

Neisseria meningitidis (also referred to as meningococcus) is a Gram-negative bacterium that is an obligate commensal of the human nasopharyngeal mucosa. Meningococci can cause fulminant, rapidly fatal sepsis and can cross the blood–meningeal barrier, causing meningitis. We have recently shown that virulent encapsulated bacteria first adhere to endothelial target cells through their type IV pili and then proliferate, locally forming a colony at their site of attachment on the cell surface. Adhesion then promotes the local formation of membrane protrusions that surround bacteria favoring bacterial internalization within intracellular vacuoles and their transcytosis (Nassif et al., 2002). The formation of membrane protrusions stems from the organization of specific molecular complexes involving the molecular

linkers ezrin and moesin (known as ERM [ezrin-radixin-moesin] proteins), along with the clustering of several membrane-integral proteins, including CD44, intracellular adhesion molecule (ICAM) 1, and cortical actin polymerization (Hoffmann et al., 2001; Eugene et al., 2002; Lambotin et al., 2005).

Recruitment of blood leukocytes to the site of infection involves a sequential, multistep process, from the tethering of leukocytes, followed by their arrest on the surface of activated endothelium to transendothelial migration. Leukocyte arrest, or firm adhesion, is mediated by the interaction of endothelial vascular cell adhesion molecule (VCAM) 1 with the integrin $\alpha_4\beta_1$ (VLA-4) and of ICAM-1 and -2 with the integrins $\alpha_1\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1). Shortly after arrest, most leukocytes spread and begin to migrate laterally over the apical surface of the endothelium to reach the nearest intercellular junction, a step referred to as locomotion (Schenkel et al., 2004). Leukocytes then migrate through the endothelial cell junctions (diapedesis or paracellular migration) or transmigrate directly through

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Abbreviations used in this paper: ERM, ezrin-radixin-moesin; HBMEC, human bone marrow endothelial cell line; ICAM, intracellular adhesion molecule; SDF, stromal cell-derived factor; VCAM, vascular cell adhesion molecule.

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individual endothelial cells (transcellular migration; Carman and Springer, 2004; Cinamon et al., 2004; Yang et al., 2005).

Active roles of the endothelium in facilitating leukocyte extravasation have been suggested by a variety of studies (Cook-Mills and Deem, 2005; Millan and Ridley, 2005). We and others have shown that endothelial adhesion molecules are involved in transducing leukocyte adhesion-mediated signaling responses to endothelium, leading to actin cytoskeletal reorganization (Etienne-Manneville et al., 2000; Cook-Mills et al., 2004). Moreover, recent studies have demonstrated that leukocyte adhesion promotes the remodeling of the apical endothelial plasma membrane into projections that surround adherent leukocytes (Carman and Springer, 2004; Shaw et al., 2004; Barreiro et al., 2005). These structures, referred to as endothelial docking structures or transmigratory cups, are essential to promote firm adhesion and extravasation of leukocytes through paracellular as well as transcellular routes. These docking structures result from the dynamic redistribution of VCAM-1 and ICAM-1 at the leukocyte–endothelial contact area, accompanied by the recruitment of activated ERM proteins and by cortical actin polymerization. Extravasation is therefore an active, sequential process that requires drastic morphological changes involving the clustering of adhesion receptors on both leukocytes and endothelial cells.

Because *N. meningitidis* adhesion, like leukocyte adhesion, induces important cytoskeletal modifications at the endothelial surface, we analyzed the consequences of bacterial infection on leukocyte extravasation. Our results provide evidence that *N. meningitidis*, by recruiting the ERM proteins ezrin and moesin, prevents the formation of the endothelial docking structures that are crucial in providing directional guidance for leukocyte emigration.

Results

Infection of human endothelial cells by *N. meningitidis* affects leukocyte adhesion and diapedesis

To investigate whether leukocyte diapedesis process was altered by infection of human endothelial cells with *N. meningitidis*, we analyzed the interaction of freshly isolated human monocytes or neutrophils with infected monolayers of a human bone marrow endothelial cell line (HBMEC). Endothelial cell monolayers, either untreated or preactivated with 100 U/ml TNF- α for 24 h, were left uninfected or were infected with a pilated encapsulated strain of *N. meningitidis* (2C43 strain) for 1 h, during which small bacterial colonies developed on the cell surface of ~30–40% of the total monolayer.

Leukocyte adhesion to infected endothelial cells was assessed by measuring their attachment under flow conditions (Fig. 1 A). As expected, when a shear stress was applied (20 ml/h; 0.88 dyn/cm²), both monocytes and neutrophils adhered poorly to unactivated HBMECs, whereas they attached to endothelial cell monolayers preactivated by TNF- α treatment. Interestingly, infection of the endothelial cells by *N. meningitidis* promoted adhesion of leukocytes to unactivated monolayers but did not further increase leukocyte adhesion to TNF- α -activated mono-

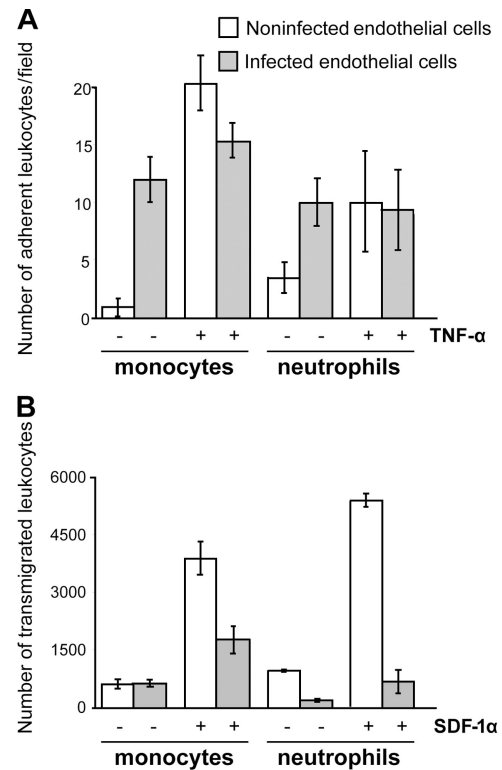


Figure 1. Infection of human endothelial cells by *N. meningitidis* induces leukocyte firm adhesion under flow but prevents their transendothelial migration. (A) HBMECs, activated or not with TNF- α (100 U/ml; 24 h), were either not infected or infected for 1 h with *N. meningitidis*. Freshly isolated monocytes or neutrophils were then allowed to adhere for 1 h under flow conditions (20 ml/h; 0.88 dyn/cm²). The number of adherent leukocytes was quantified by direct visualization. Mean values (\pm SD) from one representative experiment out of three independent experiments performed in triplicate are presented. (B) HBMECs were grown on Transwell filters at confluence and then activated with TNF- α (100 U/ml; 24 h). Cells were either not infected or infected for 1 h with *N. meningitidis*, and freshly isolated monocytes or neutrophils were added and allowed to transmigrate for 3 h in the presence or absence of 25 ng/ml SDF-1 α in the lower chambers. In such conditions, ~30% of leukocytes adhere on both noninfected and infected monolayers. The number of transmigrated leukocytes was quantified by flow cytometry. Mean values (\pm SD) from one representative experiment out of five independent experiments performed in triplicate are presented.

layers. These results indicate that infection of endothelial cells by *N. meningitidis* promotes strong adhesion by leukocytes, similar to the firm adhesion induced upon endothelial activation by inflammatory cytokines.

Leukocyte diapedesis through monolayers of TNF- α -activated endothelial cells infected by *N. meningitidis* was then assessed. In the absence of the chemoattractant, stromal cell-derived factor-1 (SDF-1 α /CXCL12), a minimal migration of monocytes or neutrophils across noninfected endothelial monolayers was observed (Fig. 1 B). In the presence of 25 ng/ml SDF-1 α , the transmigration of both monocytes and neutrophils through noninfected endothelial monolayers was enhanced. Unexpectedly, this SDF-1 α -mediated transmigration was decreased by 60–90% when endothelial monolayers were infected by *N. meningitidis* on their apical surface before leukocyte–endothelial cell interaction. When *N. meningitidis* adhered to the endothelial cell surface, cell-associated bacteria promoted

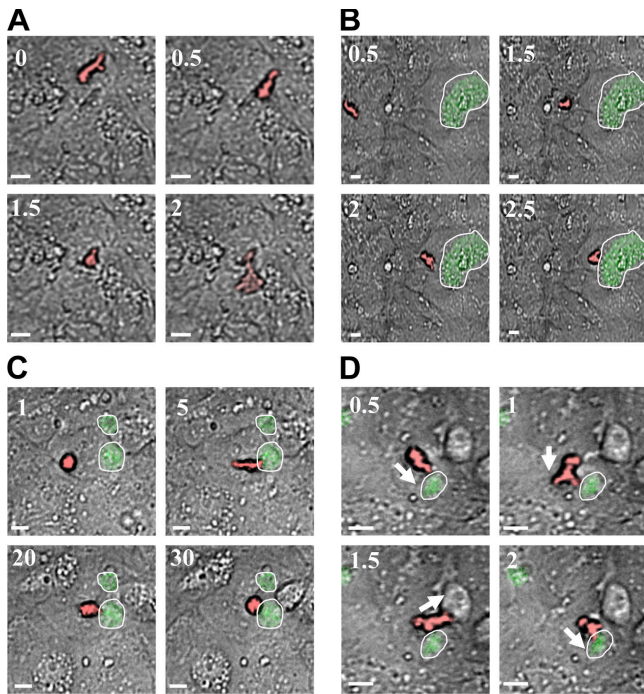


Figure 2. Live imaging of neutrophils adhering on human endothelial cells infected by *N. meningitidis*. HBMECs pretreated with 100 U/ml TNF- α for 24 h were either not infected (A) or infected for 1 h with *N. meningitidis* (B–D). Freshly prepared neutrophils were then allowed to adhere under flow conditions (20 ml/h; 0.88 dyn/cm²), and images were captured every 2 s for 30 min to make videos. For better visualization, green fluorescent bacterial colonies were surrounded with white circles and migrating leukocytes were colored in red. (A) Neutrophils adhering on noninfected endothelial cell monolayers moved within 2 min from the site of firm adhesion to the nearest intercellular junction for diapedesis. After the cell had crossed the endothelial cell monolayer, it became phase dark and continued to move underneath the monolayer (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>). (B) A neutrophil adhering to infected monolayers migrated toward a bacterial colony within 2 min, covering a distance of 90 μ m at 0.6 μ m/s (Video 2). (C) Example of a neutrophil remaining trapped for the 30-min observation period (Video 3). (D) Example of a neutrophil adhering in the vicinity of a bacterial colony, attempting to migrate in many different directions. Arrows show the direction of projections. (Video 4). Bars, 10 μ m.

the firm adhesion of leukocytes but inhibited their SDF-1 α -induced chemotaxis across a monolayer of endothelial cells.

Leukocyte motility on the apical surface of infected endothelial cells

To investigate the molecular events responsible for the blockade of leukocyte transendothelial migration, real-time microscopy experiments were performed to analyze neutrophil behavior on the apical surface of infected endothelial cells (Fig. 2). Freshly isolated neutrophils were injected under flow condition (20 ml/h; 0.88 dyn/cm²) onto TNF- α -activated HBMECs, which were either noninfected or previously infected for 1 h by *N. meningitidis*. During a 30-min period, we observed that 30–50% of the neutrophils adhering to noninfected cells rapidly moved (within 2 min) from the site of firm adhesion to intercellular junctions and underwent diapedesis (Fig. 2 A and Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>). After neutrophils had crossed through the endothelial cell

monolayer, they became phase dark and continued to move underneath the monolayer. In contrast, after initial adhesion on infected cells, \sim 90% of the neutrophils rapidly moved along the surface of the endothelial monolayers toward bacterial colonies, sometimes covering a distance as long as 90 μ m, with a mean speed of 0.6 μ m/s (Fig. 2 B and Video 2). However, once neutrophils reached a bacterial colony, their locomotion stopped and they remained trapped for the rest of the observation period (Fig. 2, C and D; and Videos 3 and 4). Interestingly, we noticed that all the trapped neutrophils were initially very active and attempted to migrate in many different directions (Fig. 2 D and Videos 3 and 4). After 15 min, these neutrophils rounded up and remained immobile, and some of them (\sim 20%) ultimately detached from the infected monolayers.

These results were further confirmed by scanning electron microscopic analysis of freshly isolated monocytes adhering for different periods of time to TNF- α -activated endothelial cells infected by *N. meningitidis* or to uninfected endothelial cells as control (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>). Although monocytes rapidly migrated underneath the endothelial monolayer after initial attachment to noninfected monolayers, few monocytes were observed underneath infected endothelial monolayers. Monocytes rather accumulated in the vicinity of bacterial colonies. No morphological changes characteristic of leukocyte phagocytic activity were observed, consistent with the reported observations that the polysaccharide capsule of virulent *N. meningitidis* strains protects the bacteria from phagocytosis by neutrophils or macrophages (van Deuren et al., 2000).

These results demonstrate that infection of endothelial cells by *N. meningitidis* induces the migration of leukocytes at the endothelial cell surface toward bacterial colonies, likely because of the release of chemotactic factors by the bacteria. Unexpectedly, the locomotion of leukocytes halted once they reached the vicinity of a bacterial colony, preventing subsequent leukocyte migration to the endothelial cell junctions.

N. meningitidis promotes the recruitment of the endothelial adhesion molecules involved in leukocyte adhesion

To unravel the molecular mechanisms mediating the blockade of leukocyte locomotion and transmigration, we examined the distribution of endothelial adhesion molecules at the surface of infected monolayers (Fig. 3 A). As we previously showed (Eugene et al., 2002), the ERM binding proteins ICAM-1 and CD44 were massively recruited to the bacterial adhesion sites, together with the ERM proteins ezrin (Fig. 3 A) and moesin (not depicted). Moreover, we observed that E-selectin, ICAM-2, and VCAM-1, which also contain an ERM binding motif in their cytoplasmic domains, were also recruited underneath bacterial colonies. 3D reconstructions indicate that all the recruited proteins concentrated in cellular projections surrounding bacteria, as illustrated for ICAM-1 (Fig. 3 B). In contrast, the junctional adhesion molecule JAM-A was not recruited at the bacteria adhesion site (not depicted), even though it is known to also play a role in leukocyte adhesion to and migration through endothelial monolayers (Bazzoni, 2003). These observations indicate

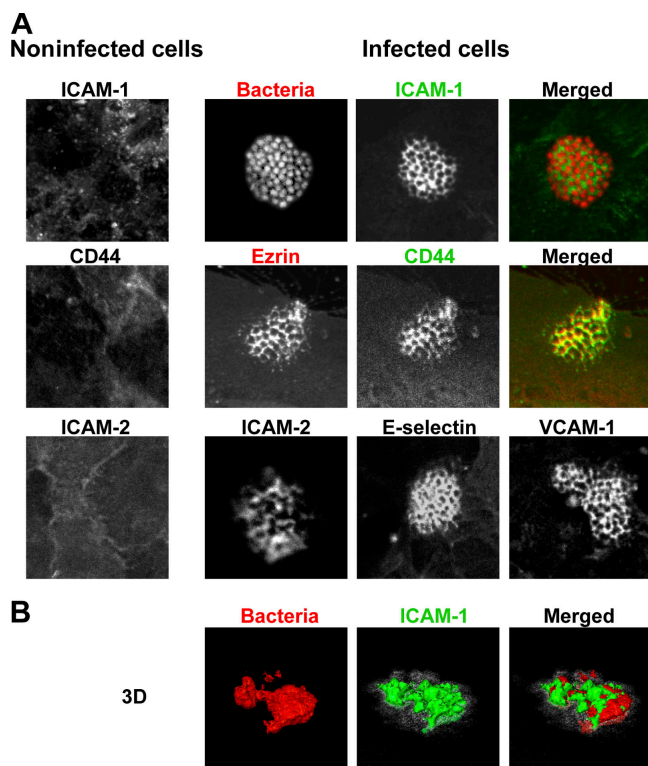


Figure 3. *N. meningitidis* recruits most endothelial cell adhesion molecules. Endothelial cells were either noninfected (left) or infected for 2 h with *N. meningitidis* (right). After fixation, cells were stained with antibodies directed against endothelial adhesion molecules and visualized by confocal microscopy. (A) Localization of ICAM-1, ezrin, and CD44 in noninfected and infected monolayers. For E-selectin, ICAM-2, and VCAM-1 staining, HBMECs were activated with 100 U/ml TNF- α for 24 h before infection by *N. meningitidis*. (B) 3D reconstructions of the recruitment of ICAM-1 (green) underneath bacterial colony (red).

that *N. meningitidis* induces the localized recruitment of most of the endothelial adhesion molecules known to be involved in leukocyte adhesion.

The cortical cytoskeleton is known to regulate the membrane localization of several adhesion molecules through one or more ERM proteins. These proteins, which are kept inactive in the cytoplasm through an intramolecular interaction, are activated by phosphatidylinositol 4,5-bisphosphate (PIP₂) binding to their NH₂-terminal domain and via phosphorylation of their COOH-terminal actin binding site by Ser/Thr kinases, such as Rho kinase (Bretscher et al., 2002; Gautreau et al., 2002). Accordingly, using a PIP₂ binding probe, the GFP-tagged PH domain of PLC δ (GFP-PLC δ -PH), we observed that infection of endothelial cells by *N. meningitidis* induced a robust production of PIP₂ at bacterial entry sites (Fig. 4 A). Moreover, an ezrin variant deleted of its PIP₂ binding site (ezrin PIP₂⁻) was not recruited but remained diffusely distributed within the cytoplasm (Fig. 4 B). Those observations clearly confirmed that ezrin recruitment at the bacteria adhesion site was dependent on PIP₂ binding. In contrast, treatment by the Rho kinase inhibitor Y-27632, which completely prevented cortical actin polymerization induced by *N. meningitidis* as previously described (Hoffmann et al., 2001), did not affect ezrin recruitment, nor did it affect the recruitment of CD44,

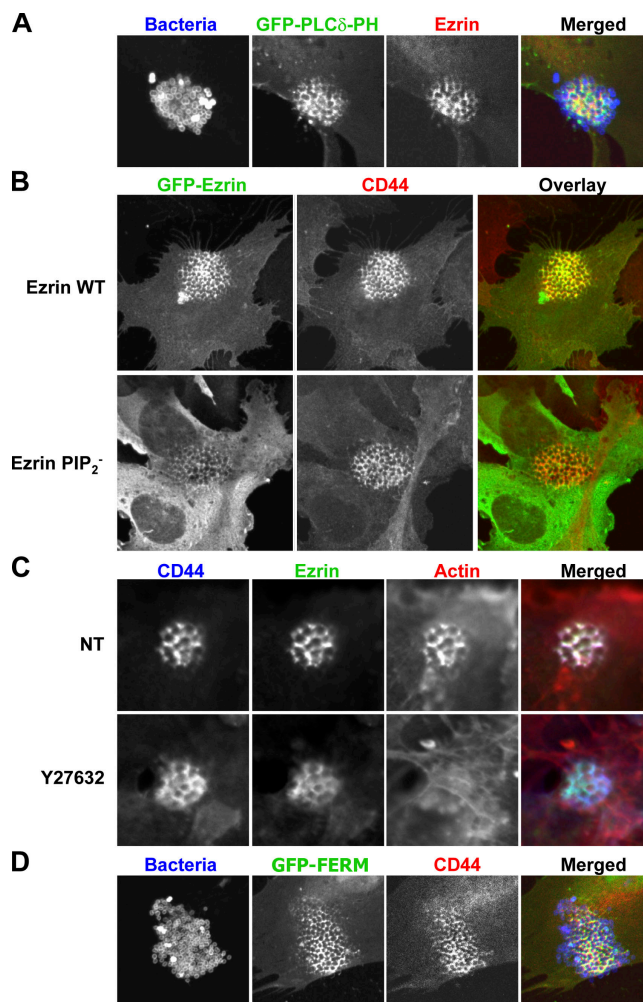


Figure 4. Recruitment of ezrin and adhesion molecules at bacterial adhesion site depends on PIP₂ binding but is independent of cortical actin polymerization. (A) HBMECs, transiently transfected with a plasmid encoding the GFP-PLC δ -PH, were infected for 2 h. Cells were then stained for bacteria (blue) and ezrin (red). (B) HBMECs were transiently transfected with plasmids encoding the wild-type (WT) ezrin (top) or the ezrin variant in which the binding to PIP₂ is abolished (PIP₂⁻; bottom) coupled to GFP. Cells were infected for 2 h and then stained for CD44 (red). (C) HBMECs were pretreated or not (NT) for 2 h with 30 μ M Y27632 and then infected for 2 h with *N. meningitidis* in the presence of the inhibitor. Cells were triple stained for CD44 (blue), ezrin (green), and F-actin (red). (D) HBMECs, transiently transfected to express the dominant-negative form of ezrin coupled to GFP (GFP-FERM), were infected for 2 h by *N. meningitidis*. Cells were then double stained for bacteria (blue) and CD44 (red). After staining, cells were analyzed by confocal microscopy. Merged images of the same fields are presented in the right panels.

ICAM-1, or VCAM-1 (Fig. 4 C). We then expressed the NH₂-terminal domain of ezrin (FERM domain), which contains PIP₂ and adhesion molecule binding sites but not the F-actin binding motif, therefore promoting a dominant-negative effect on the actin-dependent function of ezrin (Crepaldi et al., 1997). We previously showed that this ezrin domain prevents both the recruitment of endogenous ezrin and the polymerization of cortical actin induced by *N. meningitidis* (Eugene et al., 2002). We present evidence that expression of a GFP-FERM construct did not affect the recruitment of CD44 (Fig. 4 D), ICAM-1, or VCAM-1 (not depicted).

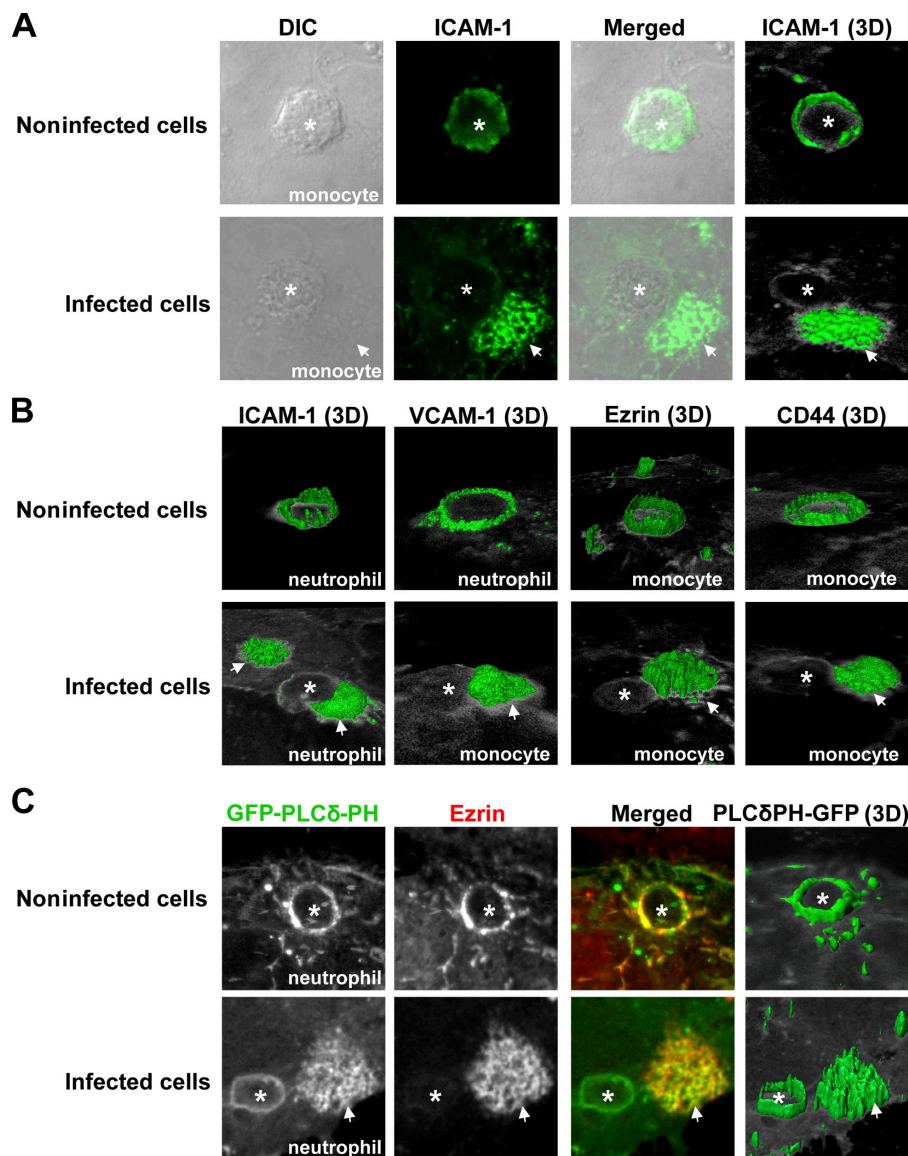


Figure 5. Infection of endothelial cells inhibits the formation of endothelial docking structures around adherent leukocytes. HBMECs were activated with 100 U/ml TNF- α for 24 h and then infected or not by *N. meningitidis* for 1 h. Freshly isolated human monocytes or neutrophils were allowed to adhere for 1 h to endothelial cells. After fixation, cells were stained with antibodies directed against endothelial adhesion molecules and observed by confocal microscopy. 3D reconstructions were built with the collected stacks. (A) Localization of ICAM-1, present in the docking structures formed around a monocyte adhering on noninfected endothelial cells (top) or underneath a bacterial colony (bottom). Note that ICAM-1 is not present around a monocyte adhering in the vicinity of bacterial colony (bottom). 3D reconstructions of ICAM-1 staining are presented in the right panels. (B) 3D reconstructions of the localization of ICAM-1, VCAM-1, ezrin, and CD44 around monocytes or neutrophils (as indicated) adhering on noninfected (top) or infected endothelial cells (bottom). Complete set of images of ezrin localization in noninfected and infected cells are provided in Videos 5 and 6 (available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>). (C) HBMECs, transiently transfected to express the GFP-PLC δ -PH, were pretreated with 100 U/ml TNF- α for 24 h and then infected or not by *N. meningitidis* for 1 h. Neutrophils were allowed to adhere for 1 h to endothelial cells. After fixation, cells were stained with antibodies directed against ezrin (red) and observed by confocal microscopy to analyze the localization of GFP-PLC δ -PH and ezrin in the docking structures formed around monocytes and neutrophils adhering on noninfected (top) or infected (bottom) endothelial cells. 3D reconstructions of the localization of GFP-PLC δ -PH are presented in the right panels. Asterisks and arrows indicate the localization of adherent leukocytes and bacterial colonies, respectively.

Collectively, these results demonstrate that recruitment of ICAM-1, ICAM-2, VCAM-1, or CD44 by *N. meningitidis* does not require an active cytoskeletal-driven mechanism. Their recruitment most likely depends on their interaction with ezrin and moesin, which are both massively recruited at the site of bacterial adhesion by a PIP₂-dependent mechanism. These events provide an efficient sequestration of the endothelial adhesion molecules beneath bacterial colonies, raising the question of whether this process affects the leukocyte diapedesis process.

***N. meningitidis* infection of human endothelial cells inhibits leukocyte diapedesis by preventing the formation of endothelial docking structures**

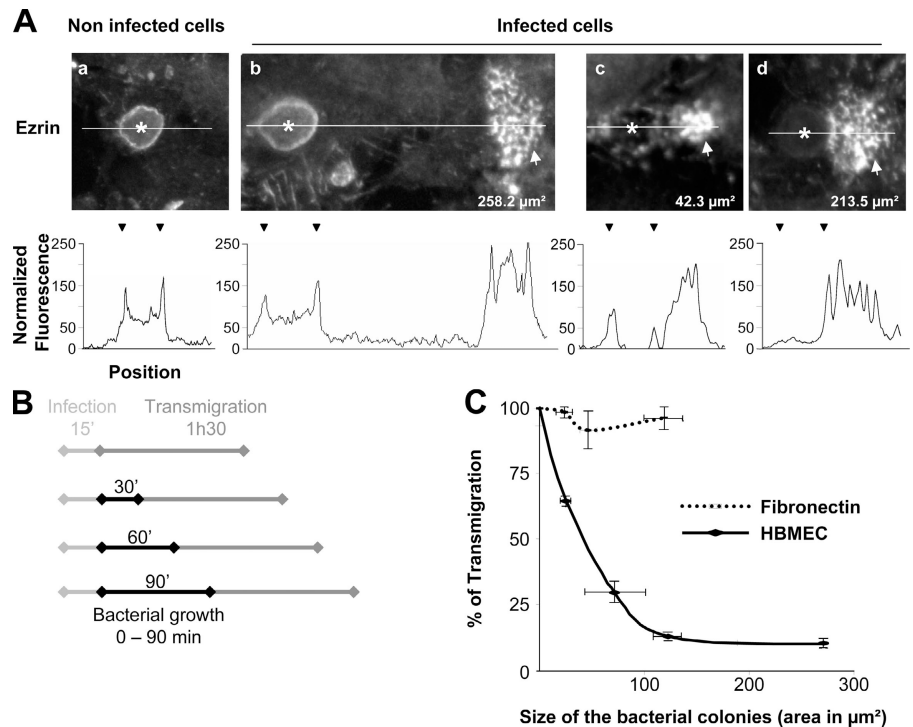
Some recent studies have highlighted a crucial role of the redistribution of ICAM-1, VCAM-1, ezrin, moesin, and F-actin at leukocyte–endothelial contacts for the formation of endothelial docking structures that prevent leukocyte detachment by shear stress and promote leukocyte transmigration (Millan and

Ridley, 2005). In agreement with these studies, we observed that both monocyte and neutrophil adhesion to noninfected HBMEC promoted the clustering of ICAM-1, VCAM-1, and ezrin as well as cortical actin polymerization around attached leukocytes. These clusters of adhesion molecules were assembled into endothelial docking structures, as assessed by 3D reconstructions of the horizontal sections (Fig. 5, A and B, top; and Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>). Staining with antibodies directed against other endothelial adhesion molecules also revealed a considerable enrichment in CD44 and ICAM-2. Moreover, in line with previous studies suggesting that PIP₂ was required for the generation of these docking structures (Barreiro et al., 2002), we noticed a strong accumulation of the PIP₂ binding probe GFP-PLC δ -PH within these structures (Fig. 5 C, top).

Strikingly, the clustering of ICAM-1, VCAM-1, CD44, and ezrin was no longer observed around leukocytes that were in contact with a bacterial colony, whereas these proteins were highly enriched in the cellular projections surrounding bacteria

Figure 6. Inhibition by *N. meningitidis* of the formation of endothelial docking structures and of leukocyte diapedesis correlates with the size of bacterial colonies.

(A) Fluorescence quantification of ezrin localization in the docking structure around a neutrophil adhering on noninfected monolayer (a), around a neutrophil interacting on a noninfected endothelial cell adjacent to infected cells (b), or around neutrophils adhering at the vicinity of a small (c) or a large (d) bacterial colony. The white line represents the z axis for the fluorescence quantification (position). The size of the bacterial colonies (area in μm^2) was determined using ImageJ software. Asterisks and arrows indicate the localization of adherent neutrophils and bacterial colonies, respectively. (B and C) HBMECs were grown to confluence on Transwell filters and treated for 24 h with 100 U/ml TNF- α . As control, Transwell filters were coated with 10 $\mu\text{g}/\text{ml}$ fibronectin for 1 h. Filters covered with HBMECs or fibronectin were either noninfected or were overlaid for 15 min with bacterial inoculum and then washed three times to remove nonadherent bacteria, and bacterial growth was allowed to proceed for the indicated period of time. Filters were then washed twice, 1.5×10^5 neutrophils were added in the upper chambers, and 25 ng/ml SDF-1 α was added in the lower chambers. After 1.5 h, transmigrated neutrophils were recovered from the lower chamber and the number of transmigrated cells was quantified by flow cytometry. The percentage of neutrophil transmigration through infected monolayers (solid line) or through cell-free fibronectin-coated filters (dotted line) was determined as the number of neutrophils that had transmigrated through infected monolayers compared with the number of neutrophil that had transmigrated through noninfected monolayers $\times 100$. Transwell filters were fixed, cells were stained with antibodies directed against bacteria, and the size of the bacterial colonies (area in μm^2) was determined using ImageJ software. (C) The histogram represents the percentage of neutrophil transmigration in relation to the size of the bacterial colonies (area in μm^2). Mean values (\pm SD) are presented from one representative experiment out of three independent experiments performed in triplicate.

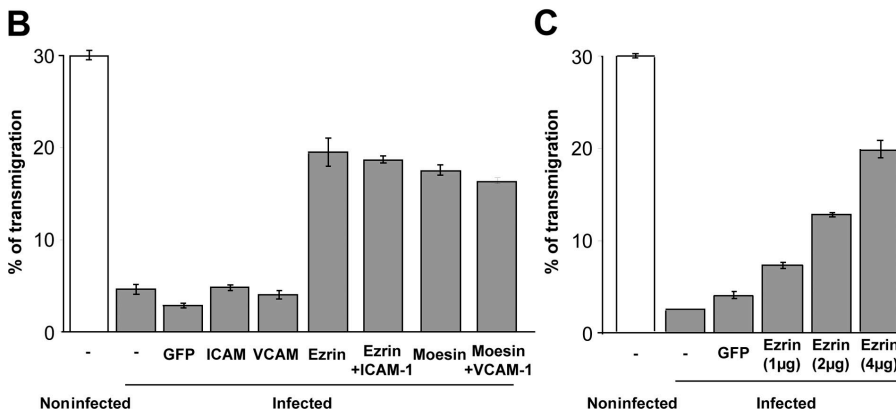
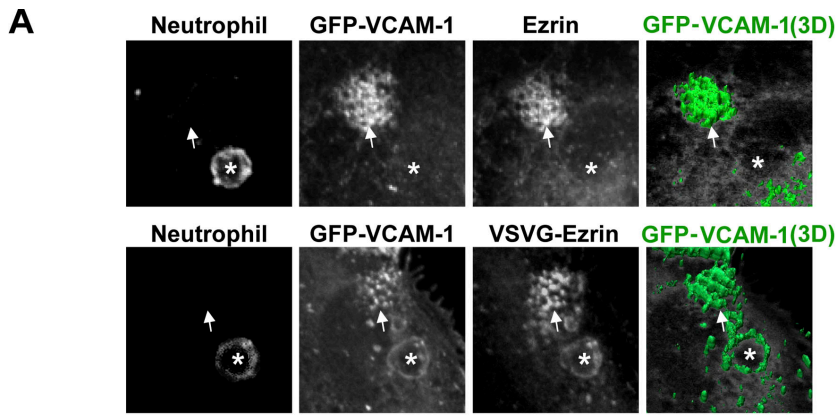


(Fig. 5, A and B, bottom; and Video 6, available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>). Interestingly, PIP₂ formation was observed around leukocytes in contact with a bacterial colony, as shown by the accumulation of GFP-PLC δ -PH (Fig. 5 C, bottom). This result strongly suggests that the lack of ezrin recruitment at leukocyte–endothelial contacts was not due to any bacterial inhibitory signal that would alter leukocyte-induced intracellular signaling in endothelial cells. These observations were further confirmed by a quantitative analysis of ezrin accumulation at leukocyte–endothelial contacts (Fig. 6 A). Interestingly, we noticed that endothelial docking structures still partially formed around leukocytes adhering at the vicinity of small bacterial colonies (Fig. 6 A, c) but not around leukocytes adhering at the vicinity of large colonies (Fig. 6 A, d), suggesting that the level of inhibition of these structures might be correlated with the size of bacterial colonies. By performing neutrophil transmigration experiments at different time points after bacterial adhesion to endothelial cells, corresponding to different sizes of bacterial colonies, we indeed clearly established that only large colonies ($>100 \mu\text{m}^2$) totally prevented neutrophil diapedesis (Fig. 6 C). Importantly, leukocytes interacting with noninfected endothelial cells adjacent to infected cells induced the formation of endothelial docking structures (Fig. 6 A, b) as efficiently as leukocytes adhering on noninfected monolayers (Fig. 6 A, a). Moreover, no inhibition of neutrophil transmigration through a fibronectin matrix was observed even when large bacterial colonies were grown on this matrix

(Fig. 6 C, dotted line). These control experiments indicated that bacteria did not directly affect the intrinsic migratory response of neutrophils. Although we cannot formally exclude the possibility that *N. meningitidis* partially interferes with leukocyte diapedesis by a bacterial chemotactic signal, our data demonstrate that the inhibition of leukocyte diapedesis is due to the absence of endothelial docking structures around the adherent neutrophils in contact with colonies. The level of inhibition is correlated with the size of bacterial colonies, strongly suggesting that *N. meningitidis* titrates away some of the key endothelial mediators of leukocyte adhesion and guidance normally present in the endothelial docking structures.

Ezrin and moesin are the key components required for endothelial docking structure formation and leukocyte diapedesis and are titrated away by *N. meningitidis*

To test this hypothesis, we assessed by immunofluorescence analysis and 3D reconstructions whether overexpression in endothelial cells of any known component of the docking structures (ICAM-1, VCAM-1, ezrin, or moesin) would rescue their formation at the surface of infected cells. No rescue was observed after overexpression of GFP-VCAM-1 (Fig. 7 A, top) or GFP-ICAM-1 (not depicted). However, when cotransfected with VSVG-ezrin, GFP-VCAM-1 (Fig. 7 A, bottom) or GFP-ICAM-1 (not depicted) now appeared within docking structures on infected cells. In addition, overexpression of GFP-ezrin



neutrophils was quantified by flow cytometry. The percentage of neutrophil transmigration was determined as the number of neutrophils that had transmigrated through infected monolayers compared with the number of neutrophils added in the upper chambers. Mean values (\pm SD) from one representative experiment out of four independent experiments performed in triplicate are presented.

or -moesin alone rescued the formation of docking structures containing the endogenous ERM-binding proteins VCAM-1 and ICAM-1 (not depicted). These data strongly suggested that ezrin and/or moesin are required for docking structure formation and are titrated away by *N. meningitidis*, thus preventing the formation of these docking structures at the surface of infected endothelial cells.

We then assessed whether overexpression of the same proteins would rescue leukocyte diapedesis after *N. meningitidis* infection of endothelial cells. Interestingly, although transfection of endothelial cells with GFP alone, GFP-ICAM-1, or GFP-VCAM-1 had no effect, overexpression of GFP-ezrin or -moesin efficiently rescued neutrophil diapedesis (Fig. 7 B). Coexpression of GFP-ezrin with GFP-ICAM-1 or GFP-moesin with GFP-VCAM-1 did not further enhance diapedesis (Fig. 7 B). In addition, transfection with increasing amounts of ezrin cDNA (Fig. 7 C) or moesin cDNA (not depicted) clearly indicated a dose-response effect.

To further demonstrate that ERM proteins are required for the formation of docking structures and leukocyte diapedesis, we evaluated the consequences in endothelial cells of overexpression of the dominant-negative FERM domain of ezrin. Endothelial cells were cotransfected with GFP-actin and VSVG-ezrin (Fig. 8 A, top) or VSVG-FERM (bottom) before adhesion of neutrophils. We observed that the expression of the FERM domain totally prevented the formation of the docking

Figure 7. Overexpression of ezrin or moesin restores the formation of the endothelial docking structures and leukocyte diapedesis through infected endothelial monolayers.

(A) HBMECs transiently transfected to express GFP-VCAM-1 alone (top) or with VSVG-ezrin (bottom) were seeded on gelatin-coated Permax coverslips for 6 h before activation with 100 U/ml TNF- α for 24 h. Cells were then infected by *N. meningitidis* for 1 h, and freshly isolated human neutrophils were allowed to adhere for 1 h to endothelial cells. After fixation, cells were stained with antibodies directed against LFA-1 (staining neutrophils) and ezrin or VSVG and observed by confocal microscopy. 3D reconstructions were built with the collected stacks. Asterisks and arrows indicate the localization of adherent neutrophils and bacterial colonies, respectively. (B) HBMECs were either nontransfected (-) or transiently transfected to express GFP alone, GFP-ICAM-1, GFP-VCAM-1, GFP-ezrin, GFP-moesin, VSVG-ezrin plus GFP-ICAM-1, or GFP-moesin plus GFP-VCAM-1, as indicated. The percentage of positive cells for each construct varied between 70 and 95%. (C) Cells were transfected with increasing amounts of plasmids encoding GFP-ezrin (1, 2, or 4 μ g). (B and C) Cells were then seeded on gelatin-coated Transwell filters for 6 h before activation with 100 U/ml TNF- α for 24 h. Cells were then infected (gray bars) or not infected (white bars) by *N. meningitidis* for 1 h, and neutrophils were allowed to transmigrate for 3 h in the presence of 25 ng/ml SDF-1 α in the lower chambers. The number of transmigrated neutrophils was quantified by flow cytometry.

structures. Moreover, the expression of either GFP-FERM (Fig. 8 B) or VSVG-FERM (not depicted) decreased neutrophil diapedesis by 50–60%, whereas the expression of GFP alone or GFP-ezrin (Fig. 8 B) had no effect.

Altogether, our results (see Fig. 9 for summary) provide clear evidence that ERM proteins are absolutely required for the formation of endothelial docking structures and that *N. meningitidis* prevents the formation of these structures by promoting the massive recruitment of both ezrin and moesin at bacterial adhesion sites. This process leads to the sequestration of key endothelial mediators of leukocyte adhesion and guidance, such as CD44, ICAM-1, and VCAM-1, and ultimately results in a strong inhibition of leukocyte diapedesis.

Discussion

Bacteria that have coevolved with their hosts use different strategies to overcome protective host defenses, such as phagocytosis or humoral defense mechanisms, by interfering with cytokine secretion or antigen presentation or by directly inhibiting T and B cell effector functions (Hornef et al., 2002; Rosenberger and Finlay, 2003). We provide evidence that *N. meningitidis* infection of human endothelial cells interferes with leukocyte transendothelial migration by preventing the formation of the endothelial docking structures required for proper leukocyte diapedesis. This inhibitory mechanism

is likely selective of *N. meningitidis* invasion, as the infection of endothelial cells by bacterial pathogens, such as *Listeria monocytogenes* or *Staphylococcus aureus*, generally leads to the up-regulation of endothelial adhesion molecules, triggering leukocyte adhesion and transmigration (Beekhuizen et al., 1997; Krull et al., 1997). Some bacterial pathogens may, however, inhibit transendothelial migration by distinct mechanisms, such as *Streptococcus pyogenes*, which induces the degradation of Interleukin-8, a key chemokine involved in the transendothelial migration of neutrophils (Edwards et al., 2005).

The most striking result of the present study is the observation that *N. meningitidis* colonies on the endothelial surface actively recruit several adhesion molecules (ICAM-1, ICAM-2, VCAM-1, and CD44), preventing them from assembling within endothelial docking structures that were recently shown to be necessary for proper leukocyte transendothelial migration (Barreiro et al., 2002; Carman et al., 2003). As a consequence, leukocytes in the vicinity of a bacterial colony remained trapped, spinning in circles and then becoming immobilized, unable to reach endothelial junctions, whereas leukocytes adhering to noninfected endothelial cells rapidly moved to the nearest junction for diapedesis. These results are highly consistent with a previous study reporting that blocking antibodies against ICAM-1 and -2 prevent monocyte locomotion on the endothelial cell surface, causing them to repeatedly “pirouette” and cross junctions without undergoing diapedesis (Schenkel et al., 2004).

Our results reveal that bacteria-induced inhibition of the formation of endothelial docking structures is strictly dependent on the recruitment of ezrin and moesin at the site of bacterial adhesion. These ERM proteins can simultaneously bind F-actin and the cytoplasmic domains of multiple adhesion molecules through their COOH- and NH₂-terminal domains, respectively. We show that expression of the truncated NH₂-terminal domain of ezrin, which displays a dominant-negative activity on actin-dependent ezrin function (Crepaldi et al., 1997), prevents the formation of the docking structures and impairs leukocyte diapedesis. Our results further reveal that by inducing the massive recruitment of ezrin and moesin underneath bacterial colonies, *N. meningitidis* prevents their accumulation at leukocyte–endothelial cell contact area and the formation of docking structures. Overexpression of ezrin or moesin in cells infected with *N. meningitidis* was sufficient to rescue both the formation of functional docking structures and efficient leukocyte diapedesis through infected monolayers. Altogether, these results firmly establish the pivotal and redundant role of ezrin and moesin in the formation of endothelial docking structures and leukocyte diapedesis. After infection by *N. meningitidis*, ERM proteins are titrated away from the leukocyte–endothelial contact area, preventing the endothelial adhesion molecules from assembling in docking structures. It is possible, however, that a fraction of ICAM-1 and VCAM-1 molecules remains at the apical surface of infected cells and may participate in leukocyte attachment without leading to a functional interaction with the actin cytoskeleton. In addition, we cannot exclude the possibility that other leukocyte–endothelial interactions are involved in leukocyte attachment to infected cells, such as JAM-A interaction with LFA-1 (Bazzoni, 2003). It is indeed interesting to

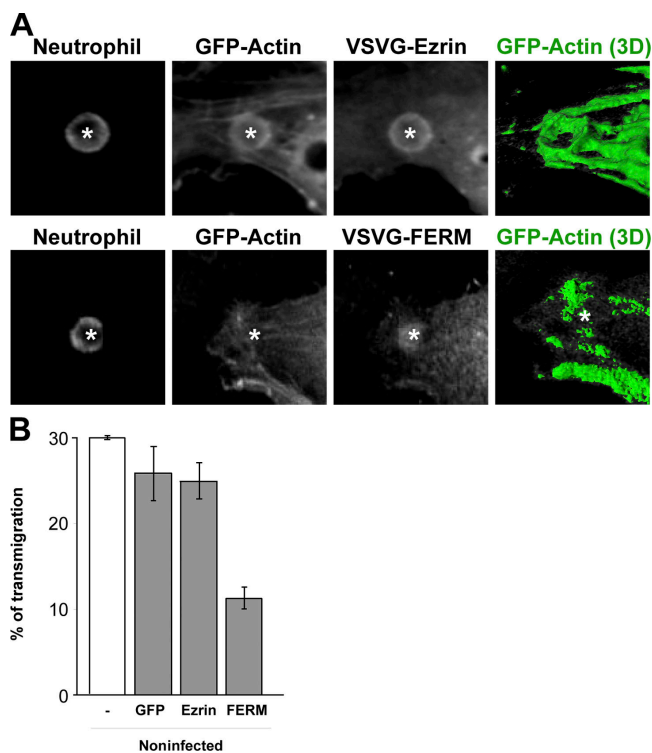
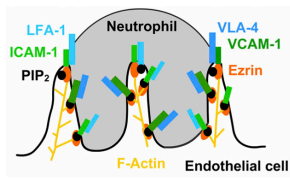


Figure 8. Overexpression of the dominant-negative NH₂-terminal domain of ezrin prevents the formation of the endothelial docking structures and leukocyte diapedesis through endothelial monolayers. (A) HBMECs transiently transfected to coexpress GFP-actin with VSVG-ezrin (top) or with VSVG-FERM (bottom) were seeded on gelatin-coated Permax coverslips for 6 h before activation with 100 U/ml TNF- α for 24 h. Freshly isolated human neutrophils were allowed to adhere for 1 h to endothelial cells. After fixation, cells were stained with antibodies directed against LFA-1 (staining neutrophils) and VSVG and observed by confocal microscopy. 3D reconstructions were built with the collected stacks. Asterisks indicate the localization of adherent neutrophils. (B) HBMECs were either nontransfected (–) or transiently transfected to express GFP alone, GFP-ezrin, or GFP-FERM, as indicated. The percentage of positive cells for each construct varied between 70 and 95%. Cells were then seeded on gelatin-coated Transwell filters for 6 h before activation with 100 U/ml TNF- α for 24 h. Neutrophils were allowed to transmigrate for 3 h in the presence of 25 ng/ml SDF-1 α in the lower chambers. The number of transmigrated neutrophils was quantified by flow cytometry. The percentage of neutrophil transmigration was determined as the number of neutrophils that had transmigrated through infected monolayers compared with the number of neutrophils added in the upper chambers. Mean values (\pm SEM) from one representative experiment out of three independent experiments performed in triplicate are presented.

note that JAM-A distribution was not altered by the bacterial infection, as JAM-A is not involved in the formation of the endothelial docking structures and seems to play a minor role in leukocyte transmigration (Shaw et al., 2004).

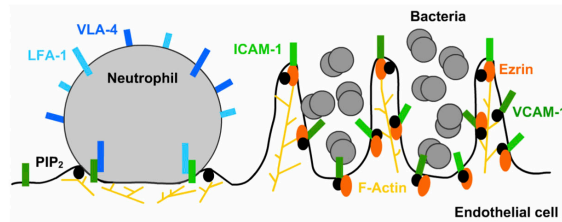
Interestingly, the same set of endothelial proteins is present in the membrane protrusions induced by *N. meningitidis* and in the docking structures promoted by leukocyte adhesion. Ezrin translocation in these two structures is dependent on PIP₂ binding, in agreement with previous studies showing that PIP₂ binding is essential for the membrane localization of ERM proteins (Barret et al., 2000; Fievet et al., 2004). However, our data shed light on some disparities in the molecular events controlling the maintenance of these two different structures. Indeed, it was previously showed that the recruitment of

**Noninfected endothelial cell:
Docking structure formation**



Transendothelial migration

**Infected endothelial cell:
Inhibition of the docking structure**



No transendothelial migration

When adhering on endothelial cells, *N. meningitidis* proliferates, locally forming a colony at the site of bacterial attachment on the cell surface. This interaction promotes the local production of PIP₂, leading to the massive recruitment of ezrin and moesin and subsequently to sequestration of ICAM-1, ICAM-2, VCAM-1, and CD44. Membrane protrusions are formed that allow bacterial internalization. When leukocytes adhere on infected endothelial monolayers, they rapidly migrate along the surface of the endothelial cells toward bacterial colonies. The sequestration of ERM proteins and of endothelial adhesion molecules by *N. meningitidis* abrogates the generation of the docking complexes around leukocytes accumulated at the vicinity of bacterial colonies. Although PIP₂ was produced at the contact area between leukocyte and infected endothelial cells, ezrin and moesin are titrated away by bacterial infection, preventing the formation of actin-rich membrane protrusions around the attached leukocytes, therefore preventing their firm attachment under flow conditions and transmigration.

VCAM-1 and ICAM-1 during leukocyte adhesion requires the polymerization of cortical actin for anchorage of the adhesion molecules at the leukocyte–endothelial contact area (Wojciak-Stothard et al., 1999; Barreiro et al., 2002). Our results further confirm that ezrin anchorage to the actin cytoskeleton is required for the formation of the endothelial docking structures. In contrast, such anchorage was not required for ezrin clustering at the bacterial interaction sites. Moreover, inhibition of the *N. meningitidis*–induced cortical actin polymerization by the Rho kinase inhibitor Y-27632 did not affect ezrin recruitment; neither did the clustering of ERM binding endothelial adhesion molecules induced by *N. meningitidis* require cytoskeletal anchoring. These observations strongly suggest an essential role of ezrin and moesin for the recruitment of multiple adhesion molecules at bacterial interaction sites by an actin cytoskeleton-independent mechanism. Interestingly, a similar mechanism of protein complex assembly was previously documented regarding the formation of the immunological synapse between T cells and antigen-presenting cells (Trautmann and Valitutti, 2003).

Systemic infection by *N. meningitidis* is a highly complex process that involves multiple interactions with host cells and host immune responses. Once meningococci have reached the bloodstream, they can cause fulminant sepsis and/or invade the meninges by crossing the blood–brain barrier. Once in the sub-arachnoid space, lacking the main humoral and cellular host defense mechanisms, bacterial proliferation can proceed in an uncontrolled manner. It is widely held that endotoxin release elicits local secretion of proinflammatory cytokines (such as TNF- α or IL-1 β), which in turn increase blood–brain barrier permeability and promote neutrophil influx. The subsequent release of neutrophil products contributes to the development of clinically overt meningitis (van Deuren et al., 2000). Because neutrophils are normally rapidly recruited to the sites of bacterial invasion and thus constitute the first line of defense against bacterial pathogens, it is tempting to speculate that the mecha-

nism described here could confer a selective advantage to meningococci invading the brain.

In conclusion, our results offer new, unexpected insights into how a bacterial pathogen may hamper the triggering of a host inflammatory response by blocking the transendothelial migration of leukocytes. Moreover, the study of this host–pathogen interaction has provided a unique window into the molecular mechanisms controlling leukocyte adhesion and transendothelial migration, thus expanding our knowledge of this fundamental cellular process.

Materials and methods

Antibodies and reagents

mAbs to ICAM-1 (11C81) and VCAM-1 (4B2) were purchased from R&D Systems, mAb to CD44 (J173) was purchased from Immunotech, mAb to E-selectin (555510) was obtained from BD Biosciences, mAb to ICAM-2 (MAB2147) was obtained from Chemicon, and mAb to LFA-1 (sc-7306) was purchased from Santa Cruz Biotechnology, Inc. Polyclonal antibodies against ezrin and moesin were kindly provided by C. Roy and P. Mangeat (Centre National de la Recherche Scientifique, Montpellier, France). Rhodamine-phalloidin was purchased from Sigma-Aldrich. The Rho kinase inhibitor Y27632 was purchased from Calbiochem. Fibronectin and human recombinant TNF- α and SDF-1 α were purchased from AbCys.

Cell culture, bacterial strain, and infection

Leukocytes were isolated from the peripheral blood of healthy volunteers by density gradient sedimentation in Ficoll (Pan Biotech), washed in PBS plus 0.1% NaCl, and resuspended at the density of 10⁶/ml in RPMI medium containing 10% SVF plus 20 ng/ml GM-CSF (R&D Systems). HBMECs (provided by B. Weksler, Cornell University, Ithaca, NY) were cultured and infected with 2C43 (formerly clone 12), a pilated capsulated Opa⁺ variant of the serogroup C meningococcal strain 8013, as previously described (Hoffmann et al., 2001; Lambotin et al., 2005).

Video microscopy and leukocyte adhesion assay under flow

Endothelial cells were seeded at 10⁵ cells/cm² on 1-well glass chamber slides (LabTek) precoated with 2% gelatin (DIFCO) and were cultured for 48 h. When indicated, cells were activated with 100 U/ml TNF- α for 12 h and then infected with *N. meningitidis* for 1 h. Live bacteria were stained for 15 min with polyclonal antibody to the bacterial capsule coupled to Cy2. The glass slide was then assembled in a parallel plate laminar flow chamber (Immunitics). A thermostated flow chamber was mounted on the

Figure 9. Remodeling of the endothelium caused by leukocyte interaction on noninfected and infected cells. When adhering on noninfected endothelial cells, leukocytes induce the remodeling of the apical endothelial plasma membrane, leading to the formation of actin-rich membrane protrusions referred to as “endothelial docking structures.” These protrusions result from the clustering of the ERM proteins ezrin and moesin at leukocyte–endothelial contact area, most likely recruited by the local production of PIP₂, and linking the transmembrane proteins ICAM-1, ICAM-2, VCAM-1, and CD44 to the actin cytoskeleton. The formation of these endothelial docking structures allows firm attachment of leukocytes under flow conditions and their proper diapedesis.

stage of an inverted microscope (Eclipse TE300; Nikon) equipped with a 20 \times objective (S fluor [Nikon]; NA of the objective lens, 0.75), and 8 \times 10⁴ leukocytes were injected using a flow of 20 ml/h (0.88 dyn/cm²) in RPMI medium at 37°C. Nomarski differential interference contrast images and fluorescence were captured every 2 s through a cooled charge-coupled device camera (CoolSNAPfx; Roper Scientific) controlled by MetaMorph software (Universal Imaging). Images were analyzed with transparencies of the labeled bacteria superimposed over QuickTime videos (Apple Computers) of the leukocytes on the surface plane of the endothelial monolayer. Videos were accelerated two times and compressed with Premiere 6.5 (Adobe). The number of adherent or transmigrated cells was quantified by direct visualization of 20 different fields (40 \times phase-contrast objective) of three independent experiments.

Cell transfection

Vectors encoding GFP-ICAM-1, GFP-VCAM-1, and GFP-moesin were provided by F. Sanchez-Madrid (Hospital Universitario de La Princesa, Madrid, Spain). Vectors encoding the VSVG-FERM, the VSVG-ezrin, the GFP-ezrin, and the GFP-tagged PIP₂⁻ ezrin mutant were provided by M. Arpin (Institut Curie, Paris, France). Vector encoding the GFP-FERM and the GFP-PLC δ -PH were provided by J. Delon and G. Bismuth (Institut Cochin, Paris, France), respectively. HBMECs were transfected using the nucleofactor system developed by Amaxa, Inc., as previously described (Lambotin et al., 2005).

Transendothelial migration assay

HBMECs were seeded on 3- μ m-pore Transwell filters (Costar) precoated with 2% gelatin at 10⁵ cells/cm², and cultured for 48 h. Cells were then treated for 24 h with 100 U/ml TNF- α . Transfected HBMECs were seeded on Transwell filters at 5 \times 10⁵ cells/cm² for 6 h and then treated for 24 h with 100 U/ml TNF- α . When indicated, Transwell filters were precoated with 10 μ g/ml fibronectin for 1 h. Filters covered with fibronectin or HBMECs were overlaid for 15 min with bacterial inoculum in starvation medium (DME supplemented with 0.1% BSA). Filters were then washed three times with starvation medium to remove nonadherent bacteria, and infection was allowed to proceed for the indicated period of time. Filters were then washed twice, and 1.5 \times 10⁵ leukocytes were added in the upper chambers. When indicated, 25 ng/ml of the chemoattractant SDF-1 α was added in the lower chambers. After 3 h, transmigrated leukocytes were recovered from the lower chambers and the number of transmigrated cells was quantified by flow cytometry. To ensure that endothelial permeability was not affected during the migration assay, cells were washed three times to remove the leukocytes and the culture medium was replaced by medium containing Evans blue. The passive diffusion of Evans blue through the monolayer over a period of 10 min was determined by densitometry analysis, and the coefficient of permeability was determined.

Confocal immunofluorescence microscopy

HBMECs were grown on Permax coverslips to confluence. After infection and leukocyte adhesion, cells were fixed and labeled as previously described (Hoffmann et al., 2001; Lambotin et al., 2005). Series of optical sections were obtained with a confocal laser-scanning microscope (TCS-SP2; Leica), using a 63 \times oil-immersion objective. 3D reconstructions were obtained using Amira software (Mercury Computer Systems) and quantification histograms with ImageJ software (NIH).

Scanning electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.066 M cacodylate, pH 7.5, for 1 h at room temperature and then rinsed twice with 0.1 M cacodylate, pH 7.5. Dehydration was performed by incubating samples with increasing concentrations of ethanol, up to 100%. Samples were submitted to critical-point drying in CO₂, sputter-coated to produce 15-nm gold particle coating, and examined using a microscope (JSM 6300F; JEOL) at an accelerating voltage of 5 kV. The number of adherent, migrating, transmigrated, or rounded cells or cells in contact with a bacterial colony was quantified by direct visualization of \sim 20 different fields, corresponding to a total of 80–100 leukocytes.

Online supplemental material

Fig. S1 shows scanning electron microscopy analysis of monocytes adhering for different periods of time on endothelial cells infected by *N. meningitidis*. Video 1 shows live imaging of neutrophil diapedesis through endothelial cell monolayer. Video 2 shows live imaging of a neutrophil migrating at the surface of an endothelial cell monolayer infected by *N. meningitidis*. Video 3 shows live imaging of a neutrophil trapped in the

vicinity of a bacterial colony. Video 4 shows live imaging of a trapped neutrophil attempting to migrate in many different directions. Video 5 shows ezrin accumulation in a 3D docking structure formed around an adherent neutrophil. Video 6 shows ezrin accumulation in the cellular projections induced by *N. meningitidis*. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200507128/DC1>.

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