

# Meningococcus Hijacks a $\beta$ 2-Adrenoceptor/ $\beta$ -Arrestin Pathway to Cross Brain Microvasculature Endothelium

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## SUMMARY

Following pilus-mediated adhesion to human brain endothelial cells, meningococcus (*N. meningitidis*), the bacterium causing cerebrospinal meningitis, initiates signaling cascades, which eventually result in the opening of intercellular junctions, allowing meningeal colonization. The signaling receptor activated by the pathogen remained unknown. We report that *N. meningitidis* specifically stimulates a biased  $\beta$ 2-adrenoceptor/ $\beta$ -arrestin signaling pathway in endothelial cells, which ultimately traps  $\beta$ -arrestin-interacting partners, such as the Src tyrosine kinase and junctional proteins, under bacterial colonies. Cytoskeletal reorganization mediated by  $\beta$ -arrestin-activated Src stabilizes bacterial adhesion to endothelial cells, whereas  $\beta$ -arrestin-dependent delocalization of junctional proteins results in anatomical gaps used by bacteria to penetrate into tissues. Activation of  $\beta$ -adrenoceptor endocytosis with specific agonists prevents signaling events downstream of *N. meningitidis* adhesion and inhibits bacterial crossing of the endothelial barrier. The identification of the mechanism used for hijacking host cell signaling machineries opens perspectives for treatment and prevention of meningococcal infection.

## INTRODUCTION

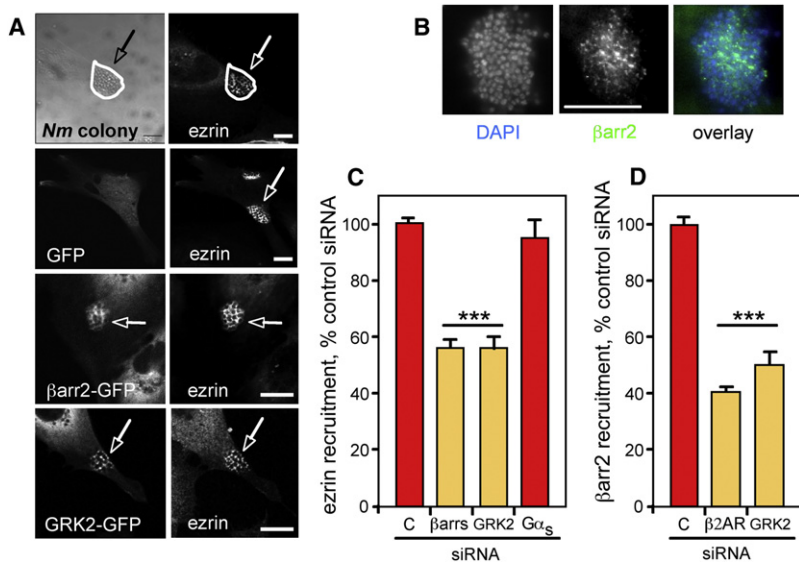
Meningococcus (*N. meningitidis*, *Nm*), a cause of epidemic meningitis and sepsis, is a commensal Gram-negative bacterium of the human nasopharynx. After bloodstream invasion, virulent encapsulated bacteria adhere to brain endothelial cells and proliferate onto the apical surface of host cells to form microcolonies at the site of initial bacterial attachment (Pron et al., 1997),

then cross the blood brain barrier (BBB) to colonize meninges (Nassif et al., 2002).

Both bacterial adhesion and subsequent *Nm*-promoted signaling in host cells depend on the presence of bacterial filamentous structures, known as type IV pili (tfp) (Nassif et al., 1994; Virji et al., 1991), and of cellular receptor(s), which still remain elusive.

In vitro experiments under controlled flow indicated that meningococcal adhesion could only occur at low shear stress levels, which are observed in capillaries upon transient reduction in flow. After initial attachment, bacteria acquire the capacity to resist higher blood velocities and to multiply, forming colonies (Mairey et al., 2006). Cellular and molecular mechanisms of this tighter adhesion are initiated by tfp-dependent activation of host cell signaling cascades. In vitro, encapsulated meningococci elicit the organization of specific “honeycomb”-shaped molecular complexes underneath bacterial colonies, referred to as “cortical plaques.” Cortical plaques result from the recruitment of molecular linkers, such as ezrin and moesin, adhesion molecules, and membrane receptors, and from localized polymerization of cortical actin, leading to the formation of microvilli-like plasma membrane protrusions among bacteria (Eugene et al., 2002), which are crucial for *Nm* colonies to resist shear stress and colonize blood vessels (Mikaty et al., 2009). Changes of the host cell cytoskeleton induced by *Nm* are associated with the turning on of multiple signaling events including the activation of the Src kinase (Hoffmann et al., 2001), the activation of the GTPases Rho, Cdc42 (Eugene et al., 2002), and Rac (Lambotin et al., 2005), the PI3-kinase-mediated recruitment of cortactin (Lambotin et al., 2005), and the activation of mitogen-activated kinases (Sokolova et al., 2004).

Whether *Nm* ultimately pass through the barrier of brain endothelial cells by transcytosis (Nassif et al., 2002) or through cell junctions has been debated for a long time. Recently, however, *Nm* were shown to recruit endothelial cell adhesion molecules, such as VE-cadherin, into cortical plaques. This recruitment leads to the formation of ectopic intercellular junctional domains at the site of bacteria-host cell interaction, depletion of junctional



**Figure 1. Requirement of GRK2 and βarrs for *Nm*-Induced Ezrin Honeycombs**

(A) hCMEC/D3 cells transfected with either GFP, βarr2-GFP, or GRK2-GFP and then infected for 2 hr with the *Nm* strain 2C43. βarr2-GFP and GRK2-GFP recruitment into cortical plaques was compared to that of endogenous ezrin labeled with appropriate antibodies (see [Experimental Procedures](#)) and analyzed by fluorescence microscopy. Arrows indicate *Nm* colonies; scale bar: 10 μm.

(B) Magnified image of endogenous βarr2 (Figure S1A) recruited to a bacterial colony (stained with diamidino-2-phenylindole, DAPI) revealed by a monoclonal anti-βarr2 antibody; every dot stained by DAPI corresponds to an individual bacterium of the colony; scale bar: 10 μm.

(C) Effect of siRNA-dependent inhibition of βarrs, GRK2, and Gα<sub>s</sub> expression (Figure S1B) on ezrin recruitment under *Nm* colonies; the recruitment assay is described in [Experimental Procedures](#). Data are the average values (± standard error of the mean [SEM]) of ezrin recruitment compared to the control (scramble siRNA-treated cells) from three independent experiments in triplicate; \*\*\*p < 0.001.

(D) Effect of siRNA-dependent inhibition of β2AR (Figure S1C) and GRK2 expression on βarr2 recruitment under *Nm* colonies; the recruitment assay is as in (C) except that the readout is based on βarr2-GFP accumulation. Data are expressed as average values ± SEM.

proteins at the cell-cell interface, opening of intercellular junctions, and subsequent crossing of the BBB (Coureuil et al., 2009).

Pleiotropic signaling events elicited by *Nm* in endothelial cells, which allow bacteria to resist blood flow and cross the BBB, might either proceed from independent parallel signaling pathways or, more likely, depend on a multimolecular signaling complex scaffolded downstream of a single host cell receptor. Among candidate proteins capable of scaffolding multiple cellular signals, β-arrestins (referred to here as βarr1 and βarr2), two ubiquitously expressed molecules initially described as regulators of G protein-coupled receptors (GPCRs), participate in many of the signaling pathways that are elicited by meningococci. For example, βarrs operate as adaptor proteins to recruit and activate Src under agonist-occupied receptors (Luttrell et al., 1999). In addition, βarrs act as scaffolds for GPCR-mediated activation of the MAP-kinases (MAPKs) ERK1/2 into cytoplasmic compartments (DeFea et al., 2000; Luttrell et al., 2001) and drive localized activation of cortical actin polymerization (Scott et al., 2006). βarrs were also shown to contribute to the activation of several Ras-family GTPases (Barnes et al., 2005; Bhattacharya et al., 2002). Finally, the junctional depletion of adhesion molecules with subsequent enhanced vascular permeability, promoted by activation of the vascular endothelial growth factor (VEGF), involves βarr-dependent endocytosis of phospho-VE-cadherin into clathrin-coated vesicles (Gavard and Gutkind, 2006).

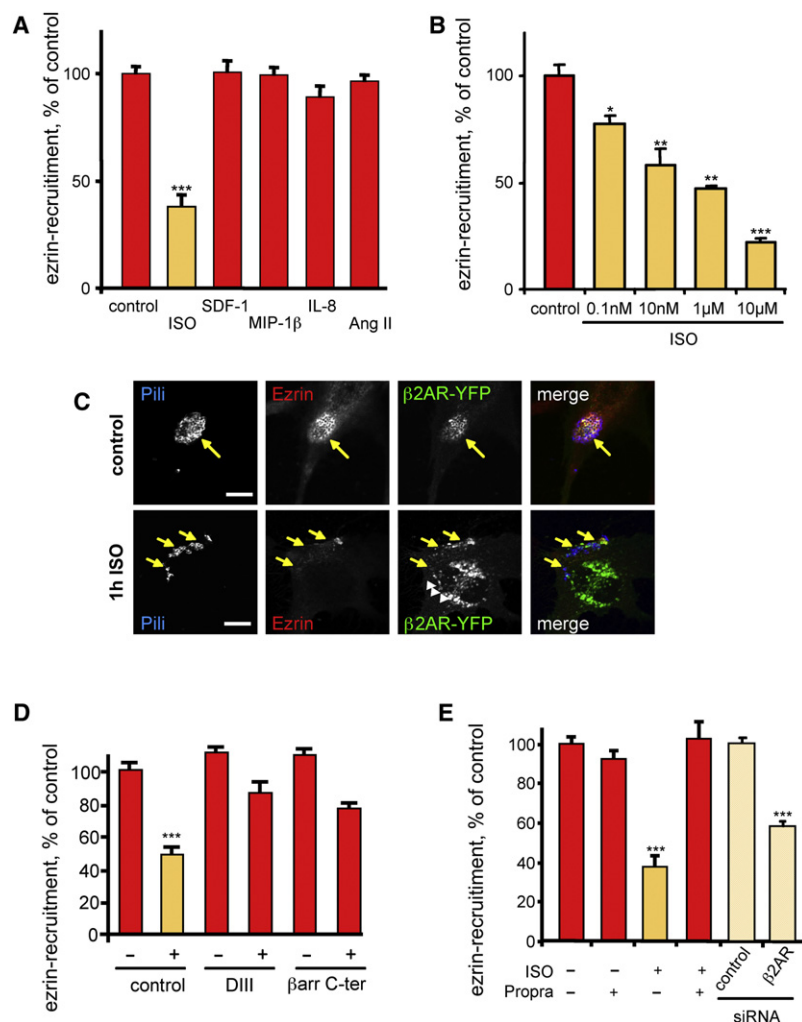
Here, we show that *Nm* colonies at the cell surface of a human brain endothelial cell line promote the translocation of βarrs to the inner surface of the plasma membrane, facing bacteria. βarrs translocated under the colonies serve as a scaffolding platform for signaling events elicited by *Nm*. Among the GPCRs expressed in the cell line, only the β2-adrenoceptor (β2AR) plays a permissive role in the formation of cortical plaques under colonies and

in bacterial crossing of cell monolayers. Our observations reveal the requisition of a β2-adrenergic/β-arrestin signaling pathway by *Nm* to promote stable adhesion onto human brain endothelial cells and subsequent crossing of the BBB.

## RESULTS

### βarrs Play an Essential Role in the Formation of Ezrin Honeycombs under *Nm* Colonies

To investigate the role of βarrs in signaling events elicited by the adhesion of *Nm* to endothelial cells, hCMEC/D3 cells, a human brain endothelial cell line, which stably maintains phenotypic features of BBB in culture (Poller et al., 2008; Weksler et al., 2005), was used as a model. Monolayers of hCMEC/D3 cells, inoculated with a capsulated piliated serogroup C *Nm* strain, displayed a characteristic reticulated immunofluorescent ezrin staining (used as a marker of cortical plaques) beneath 90% of bacterial colonies (Figure 1A). Cortical plaques were also reported to contain actin, cortactin, signaling molecules recruited in response to *Nm* adhesion (Lambotin et al., 2005), as well as components of both adherens and tight junctions (Coureuil et al., 2009). In hCMEC/D3 cells expressing green fluorescent protein (GFP)-tagged βarr2 (βarr2-GFP), βarr2-GFP colocalized with ezrin and bacteria, whereas in cells not in contact with *Nm* colonies or in noninfected monolayers, βarr2-GFP displayed its expected diffuse cytosolic distribution, indicating that bacterial colonies are capable of locally translocating βarr2. Similar results were obtained in cells expressing βarr1-GFP (not shown) and for endogenous βarr2 (Figure 1B). hCMEC/D3 cells, which predominantly express βarr2 (Figure S1 available online), were then treated with small interfering RNAs (siRNAs) to establish whether βarr translocation to bacterial colonies occurs upstream or downstream of ezrin recruitment. Reduction of both βarr1 and



### Figure 2. β2AR Involvement in Ezrin Recruitment to *Nm* Colonies

(A) Effect of pretreatment with saturating concentrations of specific GPCR agonists on *Nm*-induced ezrin recruitment in hCMEC/D3 cells (as in Figure 1C). ISO: β2AR agonist isoproterenol, 5 μM; SDF-1: CXCR4 agonist, 100 nM; MIP-1β: CCR5 agonist, 100 nM; IL-8: CXCR1-2 agonist, 100 nM; Ang II: angiotensin II, AT1R agonist, 10 nM. \*\*\*p < 0.001.

(B) Dose-dependent effect of isoproterenol on *Nm*-promoted ezrin recruitment.

(C) Effect of isoproterenol pretreatment on ezrin recruitment and β2AR subcellular distribution. hCMEC/D3 cells expressing exogenous YFP-tagged β2AR (β2AR-YFP) were preincubated with 10 μM isoproterenol (ISO) for 1 hr or left untreated, fixed, or labeled with anti-ezrin antibodies and anti-tfp monoclonal antibodies (clone 20D9) to visualize *Nm* colonies. Arrows indicate *Nm* colonies and arrowheads β2AR-containing endosomes; scale bar: 10 μm.

(D) Effect of endocytosis inhibitors on isoproterenol modulation of *Nm*-promoted ezrin recruitment. hCMEC/D3 cells were transfected with plasmids for either DIII (dominant-negative mutant of Eps15 fused to GFP) or βarrC-ter (carboxy-terminal tail of βarr fused to GFP, Figure S2E) 24 hr before isoproterenol pretreatment (+) and subsequent ezrin recruitment assay; (-) control cells pretreated with medium alone; \*\*\*p < 0.001. Representative images are shown in Figure S2F.

(E) Reversal of the effect of isoproterenol pretreatment on *Nm*-promoted ezrin recruitment by propranolol and inhibition of ezrin honeycombs by pretreating hCMEC/D3 cells with β2AR-specific siRNA (representative images in Figure S2G). ISO: 10 μM isoproterenol; Propra: 100 nM propranolol; \*\*\*p < 0.001.

In (A), (B), (D), and (E), data are expressed as average values ± SEM.

βarr2 levels, via specific siRNAs (Figure S1), led to a decrease of ezrin-containing cortical plaques in an *Nm* adhesion assay (Figure 1C). The G protein receptor kinase (GRK) family specifically phosphorylates agonist-activated GPCRs, leading to their desensitization. GPCR phosphorylation by GRKs provides docking sites for βarrs. GRK2, a member of this family, was recruited under meningococcal colonies (Figure 1A) and its depletion inhibited both ezrin and βarr2 translocation under bacteria (Figures 1C and 1D). Control siRNA or siRNA directed against another signaling molecule, the heterotrimeric Gα<sub>s</sub> protein, did not affect cortical plaque formation (Figure 1C). Altogether these data indicate that βarr translocation precedes ezrin recruitment, and they are consistent with direct or indirect activation of a cell surface GPCR following tfp-mediated adhesion to endothelial cells.

### *Nm* Adhesion to Endothelial Cells Promotes βarr-Biased β2AR Signaling

hCMEC/D3 cells naturally express several GPCRs, including β2ARs, angiotensin II AT1 receptors (P.O. Couraud, personal communication), and various chemokine receptors (Weksler

et al., 2005). Sustained stimulation with agonists is known to induce GPCR desensitization and endocytosis, thus reducing their density at the cell surface. We reasoned that, if one of these receptors was activated by *Nm* to mediate βarr and ezrin translocation to *Nm* colonies, specific ligands of the receptor might interfere with this phenomenon. Various agonists were tested using the formation of ezrin honeycombs in hCMEC/D3 cells as readout. Cells were pretreated before *Nm* inoculation with appropriate agonists for 2 hr, a condition that often induces internalization and desensitization of cognate receptors (Freedman and Lefkowitz, 1996). Among the compounds tested at saturating (10xK<sub>D</sub>) concentrations (Figure 2A), only isoproterenol, a β2AR-specific agonist, showed an effect on ezrin recruitment. Moreover, isoproterenol elicited a dose-dependent reduction in the number of ezrin-containing cortical plaques (Figure 2B). This effect was not due to a direct inhibition by isoproterenol of *Nm* growth in culture or of bacterial aggregation properties (Figures S2A and S2B). Also, isoproterenol pretreatment did not reduce bacterial adhesion in static conditions (Figure S2C).

In hCMEC/D3 cells expressing  $\beta$ 2AR fused to yellow fluorescent protein (YFP) ( $\beta$ 2AR-YFP), the formation of *Nm* colonies redistributed the receptor to ezrin-containing honeycombs (Figure 2C). In contrast, in hCMEC/D3 cells pretreated for 1 hr with isoproterenol,  $\beta$ 2AR-YFP was internalized in perinuclear endosomes and almost excluded from the small areas of residual ezrin below *Nm* colonies. To confirm that the inhibition on ezrin honeycomb formation by isoproterenol was due to  $\beta$ 2AR internalization, a similar experiment was conducted in the presence of endocytosis inhibitors (Figure 2D). The first, an inhibitor of the clathrin-dependent endocytic machinery, is a dominant-negative mutant of Eps15 (Eps15-DIII, Benmerah et al., 1999). The second consists of the isolated C-terminal tail of  $\beta$ arr, which contains clathrin- and AP2-binding sites and inhibits  $\beta$ 2AR endocytosis (Laporte et al., 1999 and Figure S2E). Supporting our hypothesis, hCMEC/D3 cells transfected with either mutant displayed a reduced effect of isoproterenol on ezrin recruitment below the *Nm* colonies.

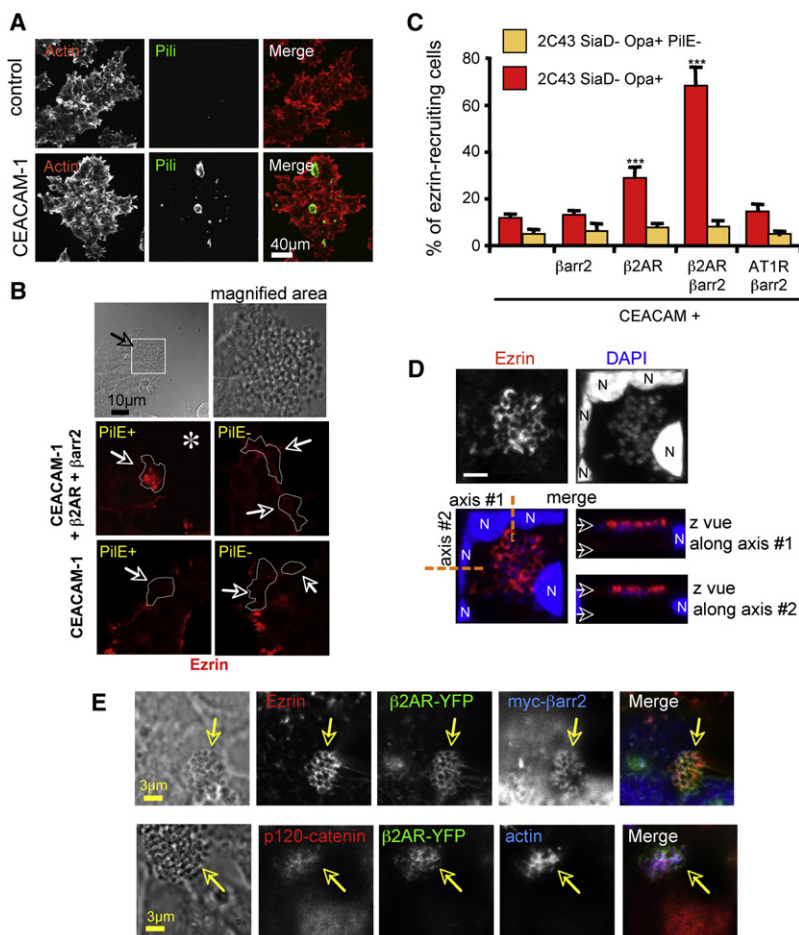
The addition during the preincubation period of propranolol, a  $\beta$ 2AR orthosteric antagonist, which inhibits receptor activation and endocytosis, blocked the effect of isoproterenol, as shown by the number of ezrin honeycombs, which remained comparable to that of untreated cells (Figure 2E). Finally, siRNA directed against the  $\beta$ 2AR in hCMEC/D3 cells significantly decreased both the number of ezrin honeycombs (Figure 2E, pale yellow histograms and Figure S1) and  $\beta$ arr2 recruitment under bacterial colonies (Figure 1D). The data above indicate that the  $\beta$ 2AR is involved in the early signaling events downstream of *Nm* adhesion.

GPCRs display various conformations, which are selectively stabilized, depending on the ligand that activates the receptor (Audet and Bouvier, 2008). Each conformation being competent for the activation of one or more effectors, different ligands can selectively activate or inhibit different subsets of effectors, leading to different signaling outputs. Activation of  $\beta$ 2ARs with catecholamines or isoproterenol, for example, stimulates both the G protein  $G_{\alpha_s}$ -dependent activation of adenylyl cyclase and the  $G_{\alpha_s}$ -independent activation of  $\beta$ arrs (Azzi et al., 2003). Activation of endogenous  $\beta$ 2AR on hCMEC/D3 cells elicited a robust stimulation of cAMP production in response to isoproterenol, monitored by a cAMP response element (CRE) luciferase reporter, which was completely blocked by propranolol (Figure S2D). Incubation of hCMEC/D3 cells with *Nm* at a multiplicity of infection (MOI) of 100 did not enhance the cAMP-dependent signal. Moreover, as the preincubation of hCMEC/D3 cells with saturating propranolol alone (Figure 2E) or knockdown of  $G_{\alpha_s}$  by siRNA (Figure 1C) had no effect on the number of ezrin-containing cortical plaques elicited by *Nm*, the *Nm*-promoted stimulation of  $\beta$ 2ARs appears to be biased toward the activation of the  $\beta$ arr pathway.

#### ***Nm*-Promoted Signaling Reconstituted in Infection-Incompetent Cells via the Expression of Exogenous $\beta$ 2ARs and $\beta$ arrs**

To obtain additional evidence that the  $\beta$ 2AR/ $\beta$ arr pathway is critical for the *Nm*-induced formation of cortical plaques in human cells, we investigated whether cells, which are not competent for the adhesion and signaling of *Nm*, can be switched to

a permissive phenotype via the expression of exogenous proteins. HEK293 cells were used as a model because they only express modest concentrations of endogenous  $\beta$ arrs (Storez et al., 2005) and no  $\beta$ 2ARs. Piliated capsulated *Nm* did not adhere onto HEK293 cells (Figure 3A) and, consequently, did not promote the formation of cortical plaques (not shown). Transfection of the  $\beta$ 2AR and/or  $\beta$ arr constructs in HEK293 cells did not restore bacterial adhesion and/or signaling (not shown), thus excluding that the  $\beta$ 2AR on its own mediates tfp-dependent adhesion of capsulated *Nm*. However, given that tfp-mediated adhesion and signaling are two independent events (Merz et al., 1999), the lack of initial bacterial adhesion could be responsible for the absence of signaling. Type IV pili are the only bacterial attributes capable of promoting adhesion of capsulated *Nm*. Yet, noncapsulated (SiaD<sup>-</sup>) strains can interact with host cells via Opa proteins, which recognize CEACAMs (carcinoembryonic antigen-related cell adhesion molecules), cellular receptor members of the Immunoglobulin superfamily (Virji et al., 1996). Being devoid of CEACAMs, HEK293 cells were thus transfected with a plasmid encoding CEACAM-1 and subsequently infected with a noncapsulated (SiaD<sup>-</sup>) Opa<sup>+</sup> derivative of the piliated capsulated serogroup C strain used in the experiments above. Although the expression of CEACAM-1 allowed the development of piliated *Nm* colonies at the surface of HEK293 cells (Figure 3A), colonies did not induce significant recruitment of ezrin (Figure 3B, left lower panel). HEK293 cells were then cotransfected with plasmids coding for CEACAM-1 and GFP (used as transfection marker) in the absence or presence of the  $\beta$ 2AR and/or  $\beta$ arr2 plasmids, and the recruitment of ezrin assessed in GFP-positive cells. In the absence of exogenous  $\beta$ 2AR and  $\beta$ arr2, only a low percentage of colonies recruited ezrin in microvilli-like structures with no visible honeycombs (Figure 3B, left lower panel and Figure 3C). Transfection of the  $\beta$ 2AR plasmid significantly raised the number of colonies ( $\approx$ 30%), which recruited ezrin in GFP-positive cells. The additional expression of exogenous  $\beta$ arr2 boosted the number of ezrin-recruiting colonies to nearly 70%, with typical honeycomb structures being visible in more than 50% of the cases (Figure 3B, left-middle panel and Figure 3C). In addition, the recruitment of ezrin was specifically promoted by the expression of the  $\beta$ 2AR, as shown by the absence of ezrin recruitment above control values with expression of the AT1 angiotensin receptor, CCR5, or CXCR4 (Figure 3C and data not shown). Interestingly, 3D reconstruction of confocal immunofluorescence images showed that ezrin accumulates both beneath and between meningococci within cellular protrusions similar to those observed in hCMEC/D3 cells infected with capsulated *Nm* (Figure 3D, Movie S1, and Movie S2). Finally, cortical plaques in HEK293 cells contained the exogenous  $\beta$ 2AR and  $\beta$ arr2 (Figure 3E) as well as endogenous actin and p120 catenin (see below). In order to verify that tfp were responsible for the formation of the cortical plaque, similar experiments were performed using a nonpiliated derivative (PiliE<sup>-</sup>) of the noncapsulated Opa<sup>+</sup> strain. This strain did not induce the formation of cortical plaques (Figure 3B, right lower and middle panels and Figure 3C). These data confirm the involvement of the  $\beta$ 2AR/ $\beta$ arr pathway in tfp-dependent signaling events, downstream of a  $\beta$ 2AR-independent adhesion of *Nm* to host cells.



**Figure 3. Reconstitution of *Nm*-Promoted Signaling in HEK293 Cells**

(A) Induction of piliated noncapsulated Opa<sup>+</sup> (2C43 SiaD<sup>-</sup> Opa<sup>+</sup>) *Nm* adhesion on HEK293 cells by expression of exogenous CEACAM-1. Forty-eight hours after transfection with a plasmid for CEACAM-1, HEK293 cells were infected with bacteria, washed, fixed, and stained with Alexa-conjugated phalloidin to reveal actin and anti-tfp antibodies.

(B) Tfp-dependent recruitment of ezrin into the cortical plaques in HEK293 cells requires the expression of beta2AR and beta2. After transfection with the indicated plasmids, cells were infected with piliated (PiliE<sup>+</sup>) or nonpiliated (PiliE<sup>-</sup>) 2C43 SiaD<sup>-</sup> Opa<sup>+</sup> strains, fixed, and labeled with anti-ezrin antibodies (lower panels). Arrows indicate bacterial colonies (limits are circled in white). Left upper panel: phase contrast image of the panel labeled with the asterisk; right upper panel: magnification of the area within the white square.

(C) Ezrin recruitment assay (as in Figure 1C) in HEK293 cells transfected with the indicated plasmids and infected with the indicated *Nm* strains. AT1R: control type 1 Ang II receptor. Bars indicate SEM from three experiments in duplicate. \*\*\**p* < 0.001. Other control receptors were tested (CCR5, CXCR4), which all failed to reconstitute ezrin recruitment above the values observed for AT1R (not shown).

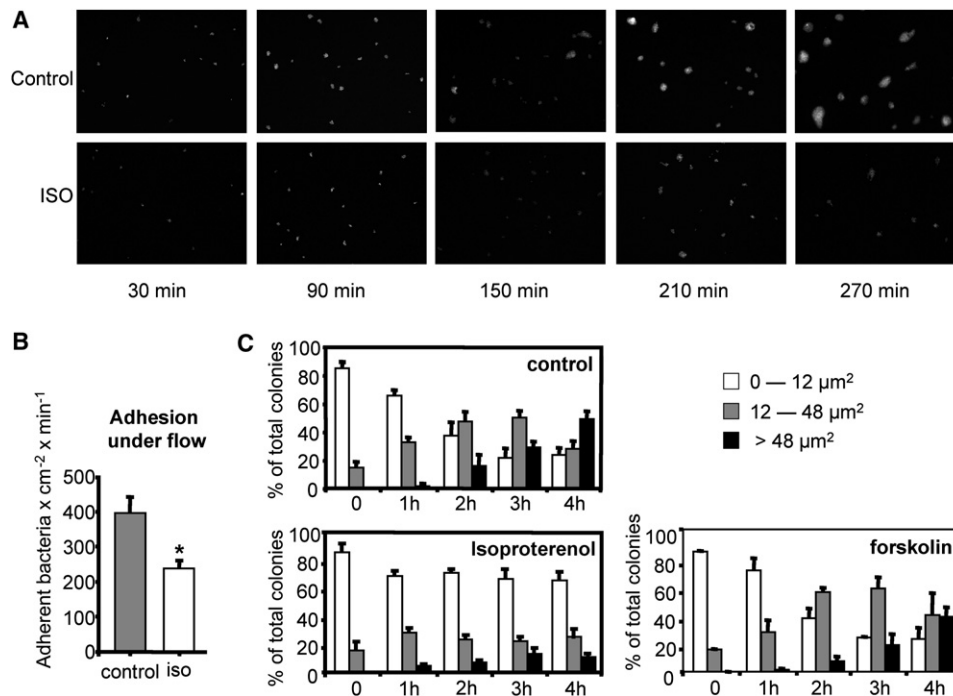
(D) Analysis of ezrin recruitment in reconstituted HEK293 cells. Cells transfected with plasmids for CEACAM-1, beta2AR, and beta2 were infected with the piliated 2C43 SiaD<sup>-</sup> Opa<sup>+</sup> strain, fixed, and stained with anti-ezrin antibodies and DAPI, and immunofluorescence was analyzed by confocal microscopy and 3D reconstruction. In the lateral views along the indicated axes, the arrows indicate the tops and the bottoms of the cells. N: DAPI-stained HEK293 nuclei. 3D-reconstituted images are shown in Movie S1. For comparison, see Movie S2 corresponding to hCMEC/D3 cells infected with wild-type *Nm*.

(E) Colocalization analysis of ezrin, p120 catenin, and actin staining with YFP-tagged beta2AR (beta2AR-YFP) or myc-epitope tagged beta2 (myc-beta2) under SiaD<sup>-</sup> Opa<sup>+</sup> *Nm* colonies (arrow) in reconstituted HEK293 cells.

### ***Nm* Resistance to Shear Stress Requires Activation of a beta2AR-Dependent Pathway**

Previous studies have shown that the bacteria-induced formation of the cortical plaque and of microvilli-like structures allow *Nm* to adhere to endothelial cells and resist drag forces generated by the bloodstream (Mikaty et al., 2009). To investigate the role of beta2AR in the stable adhesion of *Nm* under experimental conditions recapitulating the pathophysiology of bacterial adhesion to brain endothelial cells, hCMEC/D3 cells were grown on IBIDI chambers and submitted to laminar flow under an inverted microscope (Figure 4). GFP-expressing bacteria were introduced in the chamber under controlled flow, and the number and size of adhering bacterial colonies counted over time (Mairey et al., 2006). In control assays, bacterial colonies progressively increased in size, large colonies of 48 µm<sup>2</sup> or more (≥16 bacteria) representing about 50% of the total after 4 hr, whereas small colonies (≤12 µm<sup>2</sup>, about 4 bacteria) accounted for only 20% (Figures 4A and 4C). Preincubation of cells with isoproterenol for 1 hr before the perfusion of bacteria markedly reduced the average size of *Nm* colonies adhering to endothelial cells

under flow: ≈70% of the colonies remained small throughout the experiment, large colonies barely representing 10% (Figures 4A and 4C). Interestingly, whereas the numbers of colonies forming in the absence of flow were similar in control conditions and after isoproterenol pretreatment (Figure S2C), under constant flow the beta2AR agonist caused a 40% decrease in the number of colonies forming (Figure 4B). These data suggest that, because of inhibited beta-adrenergic signaling downstream of *Nm* adhesion, stable bacterial adhesion over time is impaired with larger colonies being progressively “wiped out” by the flow, a hypothesis supported by video data (compare Movie S3 and Movie S4). Thus the activation of beta2ARs appears a critical step for *Nm* to remain in strong interaction with brain endothelial cells for a sufficiently long period of time. Consistent with the involvement of a beta2AR-biased signaling pathway discussed above, preincubation with the adenylyl cyclase-stimulating agent forskolin, although inducing marked elevation of cAMP (not shown), had no significant effect on the number and size of *Nm* colonies compared to control conditions. This result also eliminates the hypothesis of a cAMP-dependent



**Figure 4. Effect of Isoproterenol on *Nm* Adhesion and Growth under Flow**

(A) Time course analysis of adhesion and growth of GFP-expressing *Nm* on confluent hCMEC/D3 cells under laminar flow. The assay is described in [Experimental Procedures](#). The time after injection of bacteria under controlled flow (0.2 dyn/cm<sup>2</sup>) is indicated below the panels. ISO indicates 1 hr pretreatment with 10  $\mu\text{M}$  isoproterenol. Fluorescent bacteria were observed using an inverted fluorescence microscope and a 20 $\times$  objective. See also [Movie S3](#) and [Movie S4](#).

(B) “Under flow” adhesion of bacteria on hCMEC/D3 cells pretreated with 10  $\mu\text{M}$  isoproterenol or left untreated. Adhesion under shear stress was determined by infecting cells grown in IBIDI flow chamber under a shear stress of 0.04 dyn/cm<sup>2</sup> as described in [Experimental Procedures](#). No significant difference was observed for static adhesion in the absence or presence of isoproterenol ( $p = 0.73$ ,  $n = 6$ ) ([Figure S2C](#)), whereas after pretreatment with the  $\beta$ 2AR agonist, “under flow” studies showed a 40% reduction of bacterial adhesion ( $*p = 0.018$ ,  $n = 6$ ). Data are expressed as average values  $\pm$  SEM.

(C) Analysis of the sizes of *Nm* colonies growing on hCMEC/D3 cells under flow in control conditions or after cell pretreatment with 10  $\mu\text{M}$  isoproterenol or forskolin for 1 hr before injection of bacteria. Colonies were classified in three groups according to their sizes for each indicated time (see [Experimental Procedures](#) for details). Data are expressed as average values  $\pm$  SEM.

modification of *Nm* adhesion to hCMEC/D3 cells that might have occurred during isoproterenol preincubation.

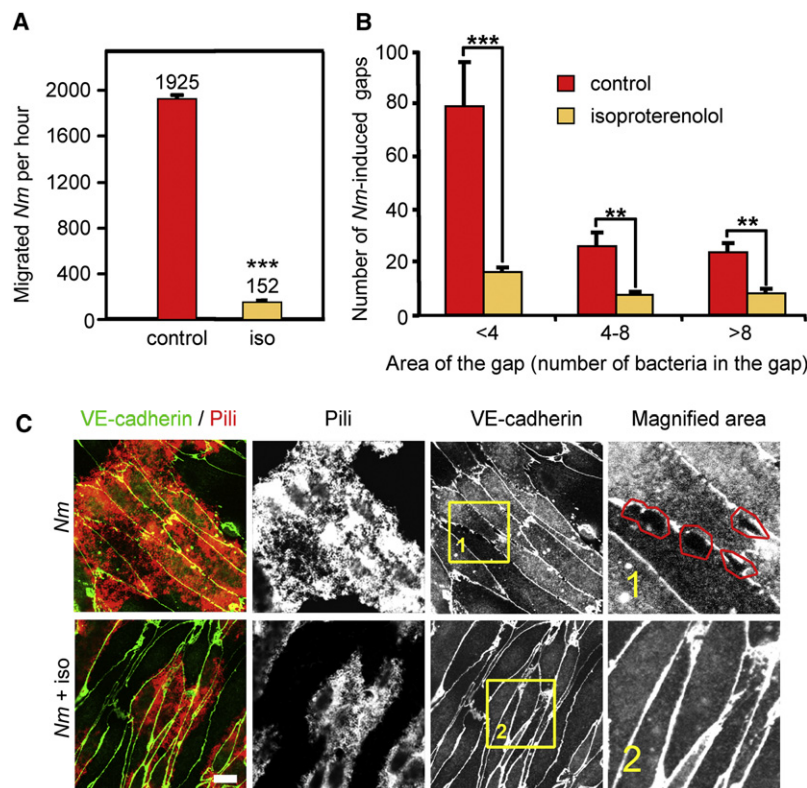
### ***Nm* Crossing of Endothelial Cell Monolayers Involves $\beta$ 2ARs**

Pili-mediated adhesion of capsulated *Nm* to brain endothelial cells results in the depletion of junctional proteins at the cell-cell interface with a subsequent opening of the intercellular junctions (Coureuil et al., 2009). This depletion is caused by the recruitment of endothelial cell adhesion molecules, such as VE-cadherin, into cortical plaques. We examined the potential role of  $\beta$ 2AR activation in these events using hCMEC/D3 cell monolayer grown on transwells as a model. First the number of bacteria passing through the monolayer was counted in control cells and in cells preincubated with isoproterenol. The  $\beta$ 2AR agonist markedly reduced the total number of meningococci in the lower chamber of transwells ([Figure 5A](#)). Gaps induced by *Nm* on cell monolayers were directly visualized by confocal microscopy ([Figures 5B and 5C](#) and [Figure S3](#)), and the area of the gaps was quantified using as surface unit the apparent diameter of a bacterium. Preincubation with isoproterenol noticeably reduced the number of the gaps of all size

([Figures 5B and 5C](#)). Together, the above data demonstrate the involvement of the  $\beta$ 2AR in *Nm* crossing of the brain endothelial barrier.

### **Multiple Roles of $\beta$ arrs in Cellular Signaling Events Promoted by *Nm***

The stabilization of bacteria at the cell surface of endothelial cells, which prevents their ejection by blood flow, requires cortactin-dependent formation of actin protrusions. Previous studies have shown that Src-dependent phosphorylation of cortactin is a key signaling intermediate in this context (Hoffmann et al., 2001). Src and activated Src (phospho-Src) accumulated below *Nm* colonies in hCMEC/D3 cells ([Figures S4A and S4B](#)). Interestingly, it was reported that recruitment and activation of Src downstream of  $\beta$ -adrenoceptors can be mediated by  $\beta$ arrs (Luttrell et al., 1999), suggesting that *Nm*-promoted activation of Src may pass via  $\beta$ arrs (see model in [Figure S4E](#)). Supporting this hypothesis, siRNAs against either the  $\beta$ 2AR or  $\beta$ arrs markedly reduced Src recruitment under colonies in hCMEC/D3 cells ([Figure 6A](#)). Docking of Src to  $\beta$ arrs involves the interaction of Src homology 3 (SH3) domains with proline residues of the consensus sequence Pro-X-X-Pro (where X represents any



**Figure 5. *Nm* Crossing of hCMEC/D3 Cell Monolayers**

(A) Transmigration assays of *Nm* on hCMEC/D3 cells grown on 3  $\mu\text{m}$  pore size MilliCell inserts. Transmigration in the presence or absence of isoproterenol pretreatment (1 hr, 10  $\mu\text{M}$ ) was expressed as the number of migrating bacteria through the cell monolayer per hour. Data correspond to five independent experiments in duplicate; \*\*\* $p < 0.001$ .

(B) Surface and number of cell-cell junction gaps induced by *Nm*, with or without isoproterenol pretreatment (10  $\mu\text{M}$ , 1 hr), visualized by confocal fluorescence microscopy on fixed MilliCell inserts immunostained for VE-cadherin. The areas of gaps (see Experimental Procedures) were classified in three groups according to the number of bacteria that can be accommodated in the gap: 4 diplococci or less, 4 to 8 diplococci, more than 8 diplococci; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ . The total surface occupied by bacteria on the apical surface of the monolayer, determined by anti-pili staining, was similar with or without isoproterenol (not shown). Data are expressed as average values  $\pm$  SEM.

(C) Imaging of *Nm*-induced gaps in cell monolayers from the experiments described in (B). Cells were stained for VE-cadherin and bacteria labeled with anti-pili antibodies. Areas beneath bacteria (yellow squares) are magnified for better visualization of the gaps (circled in red). Bar: 10  $\mu\text{m}$ . Further characterization of these gaps is shown in Figure S3.

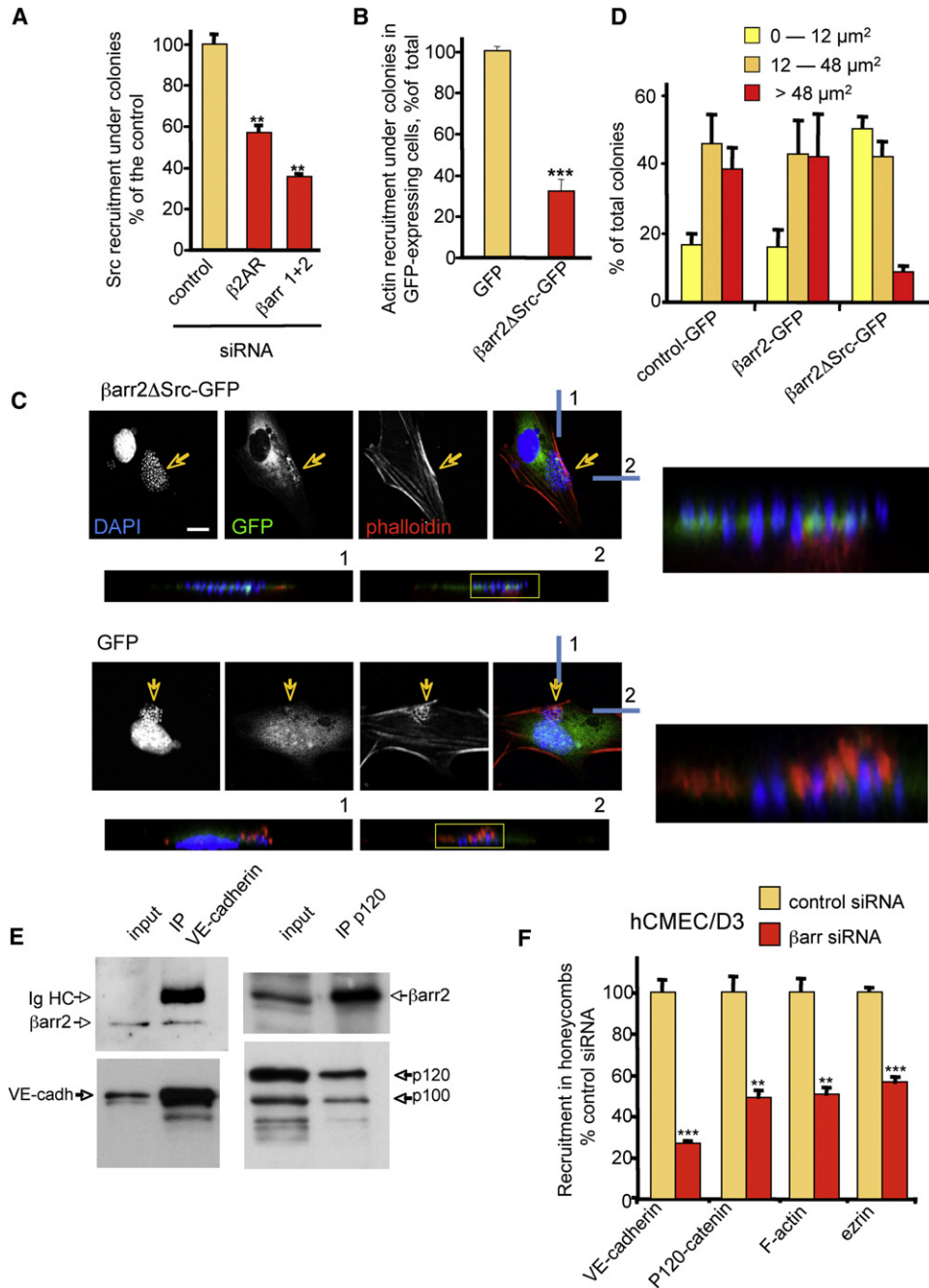
amino acid) within the  $\text{NH}_2$  terminus (positions 1 to 185) of  $\beta$ arrs. Point mutations of these proline residues lead to a dominant-negative form of  $\beta$ arr1 for Src activation, which conserved its capacity of interacting with the  $\beta$ -adrenoceptor and of mediating its endocytosis (Luttrell et al., 1999). Here we constructed the equivalent Src docking mutant in the context of  $\beta$ arr2 (myc- $\beta$ arr2- $\Delta$ Src or  $\beta$ arr2- $\Delta$ Src-GFP) and examined its effect in *Nm*-promoted phosphorylation of Src and formation of actin-rich cellular protrusions.  $\beta$ arr2- $\Delta$ Src-GFP markedly impaired recruitment of actin under *Nm* colonies in hCMEC/D3 cells, compared to control cells expressing GFP (Figure 6B). In addition, analysis of fluorescence images along the z axis confirmed the marked reduction of actin-rich protrusions among bacteria (Figure 6C). Immunoblot experiments showed a reduction of phosphorylated Src after *Nm* infection in hCMEC/D3 cells expressing the dominant-negative myc- $\beta$ arr2- $\Delta$ Src, compared to cells expressing myc- $\beta$ arr2 (Figure S4C). The dominant-negative effect of  $\beta$ arr2- $\Delta$ Src was confirmed in hCMEC/D3 cells under flow. Expression of myc- $\beta$ arr2- $\Delta$ Src displayed the same effect on *Nm* colony size as the preincubation with isoproterenol (compare Figure 6D and Figure 4C).

$\beta$ arrs might also participate in later events of *Nm* infection, namely the opening of gaps in the endothelial cell barrier (Figure S4E). This opening involves the recruitment of junctional adhesion molecules, such as VE-cadherin and p120-catenin, into the cortical plaques and their parallel depletion from endothelial cell junctions (Coureuil et al., 2009). We documented the interaction of  $\beta$ arrs with VE-cadherin and p120-catenin by coimmunoprecipitation experiments in both transfected HEK293 cells

and untransfected hCMEC/D3 (Figure S4D and Figure 6E). In addition, the reduction of endogenous  $\beta$ arrs with specific siRNAs markedly and simultaneously affected the recruitment of VE-cadherin, p120-catenin, F-actin, and ezrin into cortical plaques (Figure 6F), supporting the hypothesis that  $\beta$ 2AR-mediated activation of  $\beta$ arrs provides a molecular connection between *Nm* colonies and the recruitment of junctional molecules.

### Molecular Determinants Involved in *Nm*- $\beta$ 2AR Interaction

Among the various GPCRs expressed in hCMEC/D3 cells, only the  $\beta$ 2AR seems capable of mediating *Nm* signaling, implying some selective interaction between bacterial molecular determinants and a corresponding binding site on the  $\beta$ 2AR. The fact that tfp are necessary for reconstituting signaling events in HEK293 cells (Figure 3) indicates that the putative  $\beta$ 2AR "ligand" is present on these structures. Also in favor of this hypothesis are the consequences of the deletion of PiliV, a supposed minor pilin component, which has been shown to impair the formation of the cortical plaque (Mikaty et al., 2009), a finding confirmed in reconstituted HEK293 cells (Figure 7A). To further characterize the supposed  $\beta$ 2AR "ligand" at a molecular level, purified maltose-binding protein (MBP)-fused pilins were bound to staphylococci coated with anti-MBP antibodies (Figure 7B and Figure S5A). In addition to PiliE, two putative minor pilins, PiliV and ComP, were tested. Whereas staphylococci coated with MBP alone or MBP fused to ComP were ineffective, staphylococci carrying either MBP-PiliE or MBP-PiliV promoted  $\beta$ 2AR (Figure 7B) and  $\beta$ arr2 (Figure 7C) accumulation under staphylococcal aggregates.

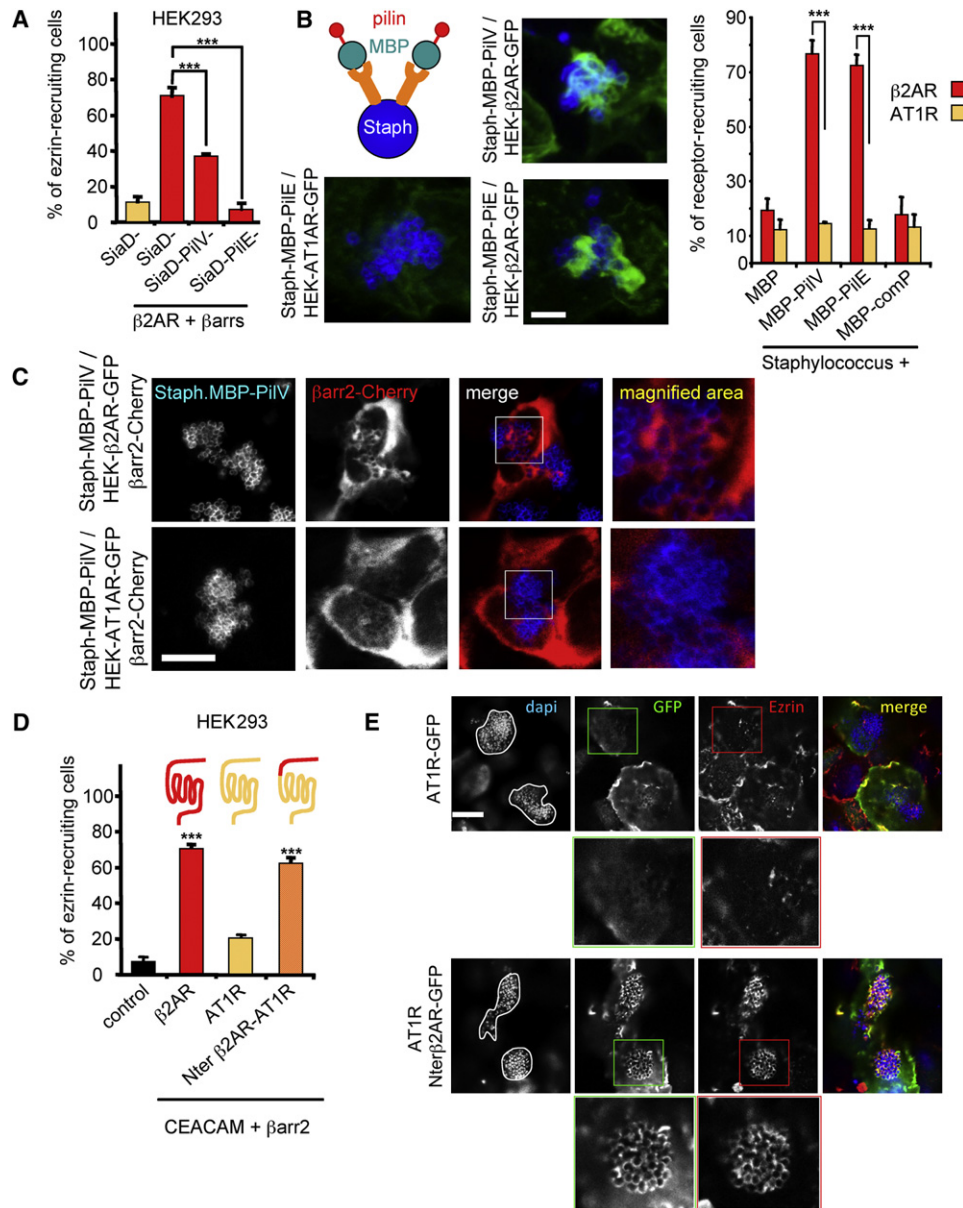


**Figure 6. Role of βarrs in Nm-Promoted Signaling**

(A–D) Docking and activation of Src (see also data in Figure S4). (A) Reduction of Src recruitment under *Nm* colonies in hCMEC/D3 cells treated with β2AR or βarr(1+2) siRNAs; n = 6, \*\*p < 0.01. (B) Dominant-negative effect of βarr2-ΔSrc-GFP on actin recruitment in the same cells. Details on βarr2-ΔSrc construct are in the Extended Experimental Procedures. n = 6, \*\*\*p < 0.001. (C) hCMEC/D3 cells transfected with plasmids coding for either βarr2-ΔSrc-GFP or GFP were infected with *Nm*. After fixation, cellular or bacterial (small dots) DNA was stained with DAPI, and actin with red-fluorescent phalloidin. Cells were examined by confocal fluorescence microscopy and Z images reconstituted along the indicated axes in cells expressing either βarr2-ΔSrc-GFP or control GFP (identified based on their green fluorescence). Magnifications of boxed areas are shown to the right. Arrows indicate the positions of bacterial colonies. (D) Sizes of *Nm* colonies growing on hCMEC/D3 cell monolayers under laminar flow (as in Figure 4) in control GFP-transfected cells, and in cells expressing either βarr2-GFP or dominant-negative βarr2-ΔSrc-GFP. Data are expressed as average values ± SEM.

(E and F) βarr-dependent recruitment of junctional proteins into cortical plaques. (E) Coimmunoprecipitation (Co-IP) experiment in hCMEC/D3 cells expressing endogenous proteins, performed with 250 μg of cell lysates; 10 μg of the input were comigrated for comparison. No signal was visible with isotopic Ig controls (not shown). (F) Effect of the siRNA-mediated inhibition of βarr1 and βarr2 gene expression on junctional and cytoskeletal protein recruitment into cortical plaques in *Nm*-infected hCMEC/D3 cells. The recruitment of indicated proteins below *Nm* colonies was analyzed as described for ezrin in Figure 1A. Data correspond to average values (±SEM) of three independent experiments in duplicate. \*\*\*p < 0.001, \*\*p < 0.01 relative to control.





### Figure 7. Molecular Determinants of *Nm*-β2AR Interaction

(A) Ezrin recruitment assay in wild-type or β2AR- and βarr-reconstituted HEK293 cells (as in Figure 3) infected with a pilated SiaD<sup>-</sup> Opa<sup>+</sup> *Nm* strain displaying normal or PilV<sup>-</sup> pili or with a nonpilated SiaD<sup>-</sup> Opa<sup>+</sup> strain (PilE<sup>-</sup>); \*\*\*p < 0.001. Data are expressed as average values ± SEM.

(B) The upper left cartoon depicts the approach to examine the respective roles of pilins. Pictures: recruitment of the indicated receptors (green) below staphylococci (blue) carrying the indicated pilins fused to maltose-binding protein (MBP) or the MBP alone (Figure S5); histograms: receptor recruitment was counted in all cells in contact with bacterial aggregates; data correspond to mean values (±SEM) of three independent experiments in duplicate. \*\*\*p < 0.001, \*\*p < 0.01.

(C) Recruitment of βarr2 fused to the Cherry protein (βarr2-Cherry) in GFP-positive (expressing the indicated receptor) HEK293 cells. The experiment was conducted as in (B) with the additional transfection of the βarr2-Cherry plasmid.

(D) Ezrin recruitment experiment comparing the β2AR, the AT1R, and the chimeric Nterβ2AR-AT1R; \*\*\*p < 0.001 compared to control and AT1R. Data are expressed as average values ± SEM.

(E) Ezrin and receptor recruitment under *Nm* colonies (circled) in HEK293 cells reconstituted with CEACAM, βarr2, and Nterβ2AR-AT1R-GFP or control AT1R-GFP. Magnification of boxed areas is shown below the corresponding panels.

In control experiments, the AT1R was not recruited to bacteria. These data suggest that PilE and PilV can function as β2AR “ligands.”

Given that *Nm* interaction with the β2AR does not involve the natural receptor binding site (data in Figure 2), the large β2AR extracellular N terminus appears a likely candidate region for

receptor interaction with the pathogen. To test this hypothesis, we constructed a chimeric Nter $\beta$ 2AR-AT1R receptor, in which the N terminus of the  $\beta$ 2AR replaced the equivalent portion of the incompetent AT1R. The chimeric receptor was indeed capable of mediating *Nm*-dependent signaling in the HEK293 reconstituted model (Figures 7D and 7E), as was the  $\beta$ 2AR. Altogether, these data suggest that pili components can directly interact with the N terminus of the  $\beta$ 2AR.

In addition to conserved biological functions, such as surface mobility, adhesion, or microcolony formation onto specific cell types, structural comparisons of the type IV pilins from different types of Gram-negative bacteria showed that all type IV pilins, despite some variably structured domains, share similar overall architecture (Craig et al., 2004). We therefore examined whether *Neisseria gonorrhoeae* (*Ng*), a close relative of *Nm* that most often causes isolated infection of the genito-urinary tract but can, in rare cases, spread into the bloodstream and colonize meninges (Martín et al., 2008), might elicit similar signaling events to *Nm* in endothelial cells. As shown in Figure S5, the principal signaling events reported here for *Nm* were also found in hCMEC/D3 cells infected by gonococci, demonstrating that the capacity of recruiting  $\beta$ -arrestins via  $\beta$ 2AR is not exclusive for *Nm* species.

## DISCUSSION

From our data, a more complete picture emerges of the signaling events elicited by *Nm* adhesion to human endothelial cells, leading to stable interaction with host cells and subsequent crossing of the endothelial monolayer. After binding onto target cells, *Nm* tfp interact with and activate the host cell  $\beta$ 2AR, leading to the subsequent recruitment of GRK2 and  $\beta$ arrestins, independently of the activation of the heterotrimeric  $G\alpha_s$  protein, and of its downstream adenylyl cyclase/cAMP pathway. The selective activation of part of the signaling pathways downstream of a given GPCR has been theorized and subsequently confirmed experimentally for synthetic ligands (Violin and Lefkowitz, 2007). However, this property, known as biased agonism, had not been described previously in a pathophysiological context.

Once translocated to  $\beta$ 2ARs,  $\beta$ arrestins mediate at least two major events facilitating *Nm* infection. First, they dock and activate Src, leading to the formation of actin-rich cellular protrusions, which protect bacterial colonies against drag forces generated by the bloodstream. Second, they provide sustained scaffolding for signaling events under *Nm*-activated  $\beta$ 2ARs, which lead to the accumulation of  $\beta$ arrestin-interacting proteins, such as VE-cadherin and p120-catenin, into cortical plates under bacterial colonies. This accumulation eventually depletes intercellular junctions, which loosen and become permeable to bacteria. Although epithelial cells are the main target of *Ng*, gonococci possibly use the same pathway to infect brain endothelial cells in vivo in the rare cases of blood dissemination. It remains to be established whether other tfp-bearing bacteria capable of forming microcolonies at the surface of endothelial cells and crossing endothelial monolayers can also hijack  $\beta$ arrestins and/or  $\beta$ 2ARs.

A few other examples of aberrant use of cellular GPCRs by pathogens to infect host cells exist, but the molecular mechanisms involved so far are quite different from those described here. For instance, *Streptococcus pneumoniae* is cointernalized

with the platelet-activating factor receptor PAFr and delivered via transcytosis to the opposite basolateral membrane of endothelial cells (Ring et al., 1998). Also, the human immunodeficiency virus, HIV, requires association with CXCR4 (Bleul et al., 1996) or CCR5 (Choe et al., 1996) chemokine receptors to infect cells. Binding of the viral gp120 to chemokine receptors induces unmasking and insertion of a fusion peptide into the target cell membrane (Doms and Moore, 2000). Before interacting with chemokine receptors, HIV needs to bind to a second receptor, CD4. It is likely that *Nm* also requires a second receptor for infection because the  $\beta$ 2AR alone is not sufficient for recapitulating the initial adhesion in the reconstituted cell model.

Our experiments have shown that both sustained adhesion of *Nm* to endothelial cells under controlled flow and their crossing of endothelial cell monolayers by opening cellular junctions are markedly inhibited by pharmacological preactivation and internalization of  $\beta$ 2ARs. Thus,  $\beta$ 2AR ligands, which induce  $\beta$ 2AR endocytosis, might be beneficial in association with antibiotics to prevent or treat meningococcal infection.

In conclusion, signaling downstream of *Nm* adhesion to human brain endothelial cells involves a biased activation of  $\beta$ 2ARs and the subsequent  $\beta$ arrestin-dependent docking and activation of Src, which in turn activate the cortactin-dependent formation of cellular protrusions. Subsequently,  $\beta$ arrestins scaffold cytoskeletal and junctional proteins under stabilized bacterial colonies, which progressively weakens the endothelial barrier until anatomical breakage occurs. This scenario represents an additional mechanism by which a pathogen hijacks cellular signaling machineries to infect target tissues. Fortunately, the signaling pathway involved in this scenario can be targeted by clinically approved drugs, which could now be tested as adjuvant therapies, not only in the context of *Nm* meningitis but also possibly for meningococcal sepsis, the most devastating form of *Nm* infection, which is characterized by diffuse endothelial leakage and subsequent irreversible shock.

## EXPERIMENTAL PROCEDURES

Extensive description of the methods can be found in [Extended Experimental Procedures](#).

### Cell Line and Bacterial Strains

The hCMEC/D3 cell line, retaining the main characteristics of primary brain endothelial cells, was described previously (Weksler et al., 2005).

A pillated capsulated Opa<sup>-</sup> variant of serogroup C meningococcal strain 8013, designated 2C43 (Nassif et al., 1993), was used throughout the study unless otherwise specified. Noncapsulated isogenic derivatives were engineered by introducing a *siaD* mutation. Isogenic nonpillated, pillated, or PiiV<sup>-</sup> variants of an Opa<sup>+</sup> noncapsulated 2C43 derivative were designated SiaD<sup>-</sup> Opa<sup>+</sup> PiiE<sup>-</sup>, SiaD<sup>-</sup> Opa<sup>+</sup>, and SiaD<sup>-</sup> Opa<sup>+</sup> PiiV<sup>-</sup>, respectively. *Nm* expressing the green fluorescent protein (GFP) were obtained by introducing in bacteria the pAM239 plasmid by conjugation (Mairey et al., 2006).

### Quantitative Analysis of Protein Recruitment under Bacterial Colonies

The recruitment efficiency was estimated by determining the proportion of colonies positive for the indicated protein of interest. At least 50 colonies were observed per coverslip. Each experiment was repeated several times in duplicate or triplicate. Data were examined for significance using Student's *t* test.

### Transfections

hCMEC/D3 cells were transfected using the Amaxa Nucleofactor Kit for Huvec (Amaxa Biosystem) or jetPEI transfection reagent (Polyplus Transfection) according to the manufacturer's instructions. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen).

### Nm Adhesion under Shear Stress

hCMEC/D3 cells were grown on disposable flow chambers (15  $\mu$ -Slide VI, IBIDI) coated with 5  $\mu$ g/cm<sup>2</sup> of rat-tail collagen type I. For experiments investigating *Nm* colony growth under shear stress, 10<sup>7</sup> bacteria were introduced into the flow chamber and allowed to adhere for 10 min without flow. Subsequently, a flow corresponding to a shear stress of 0.2 dyn/cm<sup>2</sup> was applied using a syringe pump (Harvard Apparatus). For adhesion assays under shear stress, 10<sup>7</sup> bacteria were allowed to adhere for 20 min under a shear stress of 0.04 dyn/cm<sup>2</sup>, then the chambers were washed 1 min under a shear stress of 1 dyn/cm<sup>2</sup> (Mairey et al., 2006).

### Nm Transmigration Assay

hCMEC/D3 cells were grown on 3  $\mu$ m pore size MilliCell inserts and infected at a MOI of 10, corresponding to 10<sup>6</sup> bacteria per insert. Four hours after infection, inserts were placed in a well with fresh medium. After an additional 30 min, the number of bacteria that had crossed the cell monolayer was determined by plating the content of the well and counting the number of colony-forming units.

To estimate the surface and number of macroscopic cell-cell junction openings, MilliCell inserts were fixed in 4% PFA and immunostained for VE-cadherin and actin. Several basolateral cross-sections of each insert were acquired using a Leica SP5 confocal microscope. Surface and number of gaps within the hCMEC/D3 cell monolayer were determined using Leica Application Suite-AF (LAS-AF) software. Data were expressed as the number of gaps per 4  $\times$  10<sup>5</sup>  $\mu$ m<sup>2</sup> of hCMEC/D3 cell monolayer surface. The size of gaps was determined using the apparent size of a *Nm* diplococcus as surface unit (approximately 1.7  $\mu$ m<sup>2</sup>).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and four movies and can be found with this article online at doi: 10.1016/j.cell.2010.11.035.

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