Sialic acid mediated mechanical activation of $\beta_2$ adrenergic receptors by bacterial pili

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Meningococcus utilizes $\beta$-arrestin selective activation of endothelial cell $\beta_2$ adrenergic receptor ($\beta_2$AR) to cause meningitis in humans. Molecular mechanisms of receptor activation by the pathogen and of its species selectivity remained elusive. We report that $\beta_2$AR activation requires two asparagine-branched glycan chains with terminally exposed N-acetylneuraminic acid (sialic acid, Neu5Ac) residues located at a specific distance in its N-terminus, while being independent of surrounding amino-acid residues. Meningococcus triggers receptor signaling by exerting direct and hemodynamic-promoted traction forces on $\beta_2$AR glycans. Similar activation is recapitulated with beads coated with Neu5Ac-binding lectins, submitted to mechanical stimulation. This previously unknown glycan-dependent mode of allosteric mechanical activation of a G protein-coupled receptor contributes to meningococcal species selectivity, since Neu5Ac is only abundant in humans due to the loss of CMAH, the enzyme converting Neu5Ac into N-glycolyl-neuraminic acid in other mammals. It represents an additional mechanism of evolutionary adaptation of a pathogen to its host.
Meninococcus (Neisseria meningitidis) is a human-restricted Gram-negative bacterium resident in the nasopharyngeal mucosa. If meninococci gain access to the bloodstream, they rapidly disseminate, cross the blood–brain barrier (BBB), and invade meninges, causing cerebrospinal meninigitis. In case of high bacterial load, infection can rapidly progress leading to peripheral vascular involvement and septic shock, the most severe life-threatening form being purpura fulminans.

Interaction of N. meningitidis with endothelial cells is critical for the initiation of peripheral vascular lesions and for the opening of BBB. This interaction involves long bacterial filamentous structures on the pathogen known as Type IV pili (Tfp), which mediate the attachment of virulent capsulated meninocccci to endothelial cells in vitro and in vivo. Tfp are made of the assembly in helical fibers of a core pilin subunit, PilE, and of other less abundant (minor) pilins, such as PilV, PilX, or ComP, which are structurally similar to PilE. They trigger signaling cascades in host cells leading to the stabilization of bacterial colonies at the endothelial cell surface and the subsequent translocation of bacteria through endothelial barriers. This interaction involves long bacterial colonies at the endothelial cell surface and the subsequent translocation of bacteria through endothelial barriers. 

A major unresolved question in this context is the molecular mechanism by which a GPCR can be activated by bacterial pilins to transduce a signaling cascade that is normally promoted by cognate receptor ligands. Cathelalamines and adrenergic agonists bind to and fully activate β2AR by interacting with its orthosteric ligand-binding pocket. This interaction causes receptor coupling to cognate G, protein, which activates adenylyl cyclases, and also the GPCR kinase (GRK)-dependent recruitment of β-arrestins, which scaffold signaling cascades and regulate receptor response. In contrast, meninococcal pili do not promote cAMP production in host cells and only induce a GRK-dependent activation of β-arrestins, which in turn activate a Src-cortxin pathway essential for the stabilization of bacterial colonies under blood flow, and recruit p120-catenin and VE-cadherin depleting them from endothelial junctions, causing the opening of the BBB. The activation by pili is allosteric, as it cannot be inhibited by an orthosteric blocker such as propranolol, and was reported to somehow involve the N-terminal extracellular region of the β2AR. Indeed, substituting the N-terminal region of the infection-incompetent angiotensin II receptor AT1R with that of the human β2AR produced a chimeric receptor that could be activated by meninococci in vitro, suggesting that some direct or indirect N. meningitidis interaction with the β2AR N-terminus might mediate β-arrestin-selective signaling.

Growing N. meningitidis colonies are submitted to forces exerted by blood flow. Consequently, to cope with hemodynamic forces that oppose attaching to endothelial cells at the initial stages of infection, bacterial adhesion only occurs at low levels of shear stress, which are mostly found in capillaries. Moreover, Tfp-induced signaling triggers host cell plasma membrane reorganization to form filopodia-like structures that come in close contact with bacteria, expanding the interaction surface between the colonies and the endothelium and contributing to their resistance to shear stress. In addition to their passive “hooking” role, Tfp are actively involved in the generation of mechanical forces. Early studies in Neisseria species demonstrated that pilus retraction powered by the PilT ATPase generates nanonewton forces in vivo allowing bacteria to crawl over surfaces. These forces, when applied to the cell surface via pilicoated beads mobilized by optical tweezers, were sufficient to induce ezrin recruitment under beads. Also, intermittent Tfp-dependent traction forces inside bacterial aggregates were recently reported to play a role in the adaptation of N. meningitidis aggregates to the geometry of invaded capillaries. Although Tfp need to bind to host endothelial cell to generate pulling forces, the plasma membrane tether involved in this process has not been identified yet. Previous studies in flow chambers, which recapitulate the hemodynamic conditions found in capillaries, have demonstrated that the β2AR is instrumental in the signaling process, leading to stabilizing small meninocccal colonies under flow. A potential mechanistic explanation of these observations would be that the β2AR is at the same time a mechano-sensor and a transducing receptor for meninocccal Tfp.

A further central open question related to meninococcus-induced signaling is why this pathogen only infects humans and cannot activate signals in non-human endothelial cells. Several adaptive features have been proposed to participate in the species selectivity of meninococcus, particularly when the initial bacterial load is low in vivo. Neisseria meningitidis has established specific interactions with human transferrin, which carries the iron indispensable for bacterial growth. Similarly, the interaction of meninococcus with human complement factor H protects the pathogen from the lytic action of the complement components. However, in reconstituted cell systems in which these proteins are absent, meninococcus signaling only requires the presence of the β2AR at the surface of host cells of human origin, provided that at least a surrogate adhesion system permits the initial adhesion of the pathogen. These observations suggest that the restriction of meninococcus signaling for human cells is somehow linked to the mechanism of activation of the β2AR.

Here we show that the β2AR functions as a mechano-sensor for Tfp and that its allosteric activation by meninococci is caused by mechanical traction forces. These forces are applied via two asparagine-branched glycan chains with terminally exposed N-acetyl-neuraminic acid (sialic acid (NANA), Neu5Ac) residues in the receptor extracellular extremity, independently of surrounding amino-acid residues. Because of the lack of the enzyme converting Neu5Ac into N-glycolyl-neuraminic acid (Neu5Gc), human NANA differs from that of other mammals. The requirement of Neu5Ac for β2AR mechanical activation thus appears to be essential in the pathogen selectivity for human cells and represent an additional example of evolutionary adaptation of a pathogen to its hosts.

**Results**

**Tfp activate β2AR via interaction with receptor N-glycans.** Meningococcus can activate in vitro an infection-incompetent AT1R in which the N-terminus is substituted by that of the human β2AR, suggesting that this region of the β2AR is essential for triggering receptor signaling. To further demonstrate that meninococcal pilins PilV and PilE specifically interact with the β2AR N-terminus, we used a homogeneous time-resolved fluorescence energy transfer (HTRF)–based assay in intact cells (Fig. 1a). Human HEK-293 (human embryonic kidney-293) or baby hamster kidney (BHK) cells were transfected with a construct coding for the HA epitope, followed by the first 27 amino-acid residues of the human β2AR, corresponding to the N-terminal extracellular region (EC1), the first transmembrane domain (TM1), and the first residues of the first intracellular loop, fused in phase upstream of green fluorescent protein (GFP) (identified as HA-EC1-TM1β2AR-GFP). The signal of GFP fluorescence was used to control that the expression level of this construct was equivalent in the various assays (Supplementary Fig. 1). Cells were first incubated with excess recombinant pilins containing a 6×His N-terminal tag: PilV, PilE, or ComP, a minor
pilin not involved in meningococcal signaling and used as a negative control. Anti-HA-Tag antibodies coupled to the HTRF donor and anti 6×His-Tag antibodies coupled to the HTRF acceptor were then added for 1 h before excitation and reading. In this assay a FRET signal can only be recorded if the donor and the acceptor are located at a distance of 10 nm or below. A dose-dependent specific HTRF signal was measured with recombinant His-PiIV and His-PiEL in human cells expressing controlled levels of HA-EC1-TM1β2AR-GFP (Fig. 1b, c), whereas no signal was observed with His-ComP. These data indicate a direct binding of PiEL and PiIV to the extracellular N-terminal region of the human β2AR. Since meningococcus only signals in human cells, we compared human and mouse β2AR in a reconstituted assay in vitro, in the attempt of delineating the possible role of specific amino-acid residues of β2AR N-terminus (Supplementary Fig. 2) in receptor activation by bacterial pilus. While human HEK-293 cells reconstituted with mouse β2AR could be activated by N. meningitidis in vitro, the induced signaling being illustrated by ezrin recruitment under colonies as endpoint, mouse endothelial cells expressing exogenous human β2AR were not activated by the pathogen even in the case of human CEACAM-1-promoted mock adhesion (Fig. 1d). Consistently, no specific binding signal could
be measured with the HTRF assay if the human β2AR N-terminus was expressed in hamster cells (Fig. 1b). Thus, the capacity of meningococci to bind to and activate the β2AR in human cells is independent of the amino-acid sequence and involves other factors. Since the β2AR N-terminus is glycosylated due to the presence of two asparagine-dependent glycosylation sites, we examined whether these N-glycan chains might be important for receptor activation by N. meningitidis. Alanine substitution of asparagine residues at position 6 or 15 of the β2AR, to suppress either glycan chain, dramatically reduced ezrin recruitment under meningococcal colonies to basal levels (Fig. 2a), although both receptor mutants were correctly targeted to the cell surface and responsive to agonist activation (Supplementary Fig. 3). The AT1R, which is not activated by meningococcus, only contains one glycosylation site in its terminus. Thus, in a complementary gain-of-function approach, two glycosylation consensus sequences

**Fig. 2** Role of glycan chains in meningococcus-promoted β2AR signaling. **a**, Left panel: ezrin recruitment assays were as in Fig. 1d; the percentage of ezrin-recruiting colonies was quantified in controls and in cells expressing human CEACAM-1, β-arrestins (β-arrs) and YFP-tagged forms of: wild-type β2AR, mutant β2AR lacking either N-glycosylation site (β2AR-N6A and β2AR-N15A), wild-type angiotensin AT1R, and mutant AT1R expressing either the first β2AR N-glycosylation signal (AT1R-1Nglyβ2AR), or both signals (AT1R-2Nglyβ2AR (+9)). Red drawings represent the β2AR polypeptide and its N-glycan chains. The blue diagram represents the AT1R polypeptide with its single N-glycan chain. In mutant AT1R receptors, the N-glycan chains branched on the N-glycosylation signals from the β2AR are represented in red. Histograms represent mean values ± SEM (n = 6) (***p < 0.001 control or mutants vs. wild-type β2AR, ANOVA with Bonferroni multiple comparison test; n.s.: not significant difference). Right panel: representative IF images of the experiment; arrows indicate the position of bacterial colonies; ezrin indicates the labeling with anti-ezrin antibodies and YFP corresponds to the signal of YFP-tagged receptors. **b** HEK-293 cells expressing CEACAM, β-arrestins, and the indicated receptors were prepared, infected, and processed as in **a**. Left panel: sequence alignment of receptor N-termini (constructs used in **a**, **b**). Asparagine residues used for glycan branching on wild-type β2AR are marked by stars. HA-AT1R-1Nglyβ2AR and HA-AT1R-2Nglyβ2AR correspond to AT1R mutants expressing one or both β2AR N-glycosylation signals, respectively; the numbers within parentheses correspond to the distance between N-glycosylation signals, (+9) being the distance found in wild-type β2AR N-terminus; β2AR-N6A and β2AR-N15A are mutant β2ARs lacking either N-glycosylation site. Right panel: role of the distance between N-glycosylation sites on meningococcus-promoted ezrin recruitment. Histograms represent mean values ± SEM; ANOVA with Bonferroni’s multiple comparison test: ***P < 0.001, n.s.: not significant, for the comparison with wild-type β2AR
with asparagine residues separated by nine residues as in the β2AR were created by mutagenesis of the angiotensin AT1R complementary DNA (cDNA). Co-expression of this HA-AT1R-2Nglyβ2AR (+9) mutant with β-arrestin reconstituted N. meningitidis-promoted signaling in vitro, contrasting with the lack of effect of the replacement of the AT1R N-glycosylation consensus sequence with the first of the β2AR (HA-AT1R-1Nglyβ2AR) (Fig. 2a). These data suggest that both glycan chains of the β2AR are necessary and sufficient to support receptor activation by the pathogen, whereas the activation of a plasma membrane receptor via the engagement of its glycan chain(s) usually also involve the protein backbone23. We next investigated the importance of the distance between glycan chains in receptor activation (Fig. 2b). While maintaining the first N-glycosylated site at a constant position, the second site (NGSH) was introduced in the AT1R N-terminus at a distance of 6 to 11 residues from the first site. In addition to HA-AT1R-2Nglyβ2AR (+9), only the AT1R mutant where the glycosylation sites were separated by eight residues (HA-AT1R-2Nglyβ2AR (+8)) promoted effective meningococcal activation in host cells. Altogether, these data demonstrate that Tfp-promoted signaling requires two glycan chains, at a definite distance in the N-terminus of the β2AR, independently of surrounding amino-acid residues.

**NANA residues are essential for β2AR activation.** The above data indicate that a particular distance of β2AR glycan chains is required for full interaction with meningococcal Tfp and subsequent receptor activation. Lectins were next used as inhibitors of receptor activation in an in vitro assay to identify the sugars that are specifically involved in this process (Fig. 3). This assay, performed with the same procedure as in reconstituted HEK-293 cells, was conducted instead on human hCMEC/D3 cells, which retain main features of primary brain micro-vessel endothelial cells, express endogenous CD147, β2AR, and β-arrestins, and represent a validated model of meningococcal infection8. Confirming the prominent role of β2AR in mediating meningococcal Tfp-promoted signaling, the deletion of the β2AR gene in this cell line markedly inhibited ezrin recruitment under bacterial colonies (Supplementary Fig. 4). Among 25 tested lectins, 4 displayed highly significant inhibition; 2 of them, *Maackia amaurensis*-1 (Mal-I) and wheat germ agglutinin (WGA), almost completely blocked meningococcus-induced signaling (Fig. 3a, b) in a dose-dependent manner (Fig. 3c). Control experiments were conducted to rule out the hypothesis that the lectin inhibitory effect could depend on the induction of some β2AR endocytosis (Supplementary Fig. 5). Mal-I was reported to bind to Neu5Ac α(2,3) galactose β(1,4)GlcNAc24, whereas WGA binds to both the GltNac-GlcNAc (chitobiose) core and terminal NANA25. Succiorylated WGA, which does not bind to glycans containing sialic acid25, did not inhibit meningococcus signaling (Fig. 3a, b). Moreover, in vitro meningococcal colonization of hCMEC/D3 cells under shear stress, which can be repressed by depleting the β2AR9, was also inhibited by Mal-I (Supplementary Fig. 6). This assay recapitulates one of the pathophysiological properties of meningococcus, namely the induction of a signaling pathway, which lead to the stabilization of growing colonies under shear stress. Together, the above findings indicate that NANA participates in β2AR activation by the pathogen. Consistently, selective inhibition of sialyl transferases with 3Fax-Peracetyl-Neu5Ac (3FP-Neu5Ac) was sufficient to abolish meningococcus-induced recruitment of β-arrestin-GFP (Fig. 3d, e) and downstream signaling (i.e., ezrin recruitment, Fig. 3d, f, g) in human endothelial cells. The observation that both glycan chains of the β2AR are necessary to support receptor activation and that spacing of the glycans in this context is critical (see Fig. 2) suggests that both glycan residues in question are sialylated. To address this issue, we took advantage of a trypsin cleavage site at the end of the β2AR N-terminus to measure Neu5Ac present in isolated glycosylated receptor N-terminal fragments (Fig. 4, procedure outlined in Supplementary Fig. 7). The N-termini of HA-tagged wild-type (wt)-β2AR (+11)-β2AR, N6A-β2AR, and N15A-β2AR released from trypptic digestions of transfected HEK-293 cells were immunoprecipitated with anti-HA antibody-coated beads (Fig. 4a, b). The identity of the fragments recognized by the anti-HA antibodies was confirmed by mass spectrometry (MS) (Fig. 4c). NANA present in the glycan chains were then released by heating the samples in acidic conditions and quantified by a colorimetric assay (Fig. 4d). The amount of NANA contained in the N-termini of N6A-β2AR and N15A-β2AR was roughly half of that present in wt-β2AR or (+11)-β2AR. These data strongly support the conclusion that both glycan chains at positions 6 and 15 can individually undergo terminal sialylation and that both glycan chains from wt-β2AR and (+11)-β2AR are similarly sialylated.

**NANAs participate in pathogen species selectivity.** Neu5Ac and Neu5Gc, which differ by a single oxygen atom26, are the predominant NANAs in mammals27. Neu5Ac is synthesized from Neu5Ac by the cytidine monophosphate-N-acetylmuramic acid hydroxylase (CMAH)28. Only Neu5Ac is present in humans, because of CMAH genetic inactivity26, while Neu5Gc is predominant in other mammals. To investigate whether the exposed Neu5Ac in the N-glycan chains might contribute to species specificity of meningococcal interaction with the β2AR, CMAH was deleted in C166 mouse endothelial cells (Supplementary Fig. 8) to produce a mouse cell clone that only expresses human-type Neu5Ac-containing glycans. To demonstrate the enrichment in Neu5Ac in the CMAH-knockout (KO) clone, we took advantage of WGA selectivity for Neu5Ac compared to Neu5Gc29. Precipitation assays were performed with WGA-coated agarose beads from extracts of wt and CMAH-KO cells expressing exogenous β2AR-GFP. The glycosylated form of β2AR-GFP was substantially more abundant in precipitations from C166-CMAH-KO cells providing indirect evidence that these cells contain a higher proportion of the Neu5Ac form than parental C166 (Supplementary Fig. 8e). Experiments were then performed using a piloted non-capulated Opa+ N. meningitidis strain, and CEACAM-transfected mouse endothelial cells to allow meningococcal adhesion (see Fig. 2a). In parental C166 cells expressing exogenous human YFP-tagged β2AR, the receptor was not redistributed under bacterial colonies, contrasting with the significant accumulation that could be quantified in CMAH-KO cells (Fig. 5a). Consistently, full or partial ezrin recruitment was observed in C166 clones lacking CMAH, contrasting with the absence of recruitment in the parental control C166 cell line (Fig. 5b). Furthermore, pre-incubation with the selective agonist isoproterenol, to induce endocytosis of the endogenous β2AR, inhibited this signaling, as observed previously in human endothelial cells. These data indicate that the absence of CMAH indeed contributes to the species selectivity of meningococcus for humans.

**Pulling forces applied on glycan chains activate the β2AR.** We next aimed at determining how pili binding can induce allosteric β-arrestin-selective activation of the β2AR. Bacteria growing at the cell surface of endothelial cells are permanently submitted to forces exerted by blood flow. In addition, pilus retraction, powered by the PilT ATPase, generates nanonewton forces16,17 independently of the hemodynamic flow. We hypothesized that mechanical forces applied via Tfp to receptors might produce
**Fig. 3** Lectins and sialyl-transferases inhibitors prevent meningococcus signaling. hCMEC/D3 cells were incubated for 1 h with the indicated lectins (full names in Supplementary Table 1) at a concentration of 20 µg/ml. Cells were then washed, infected with wild-type *N. meningitidis*, and processed for the analysis of ezrin recruitment under bacterial colonies. 

**a** Histograms correspond to mean values ± SEM. Data were analyzed by ANOVA with Dunnett’s multiple comparisons test. ***p < 0.001, ****p < 0.0001, control (no lectin) vs. incubation with the indicated lectin.

**b** Representative immunochemistry images. HCMEC/D3 cell nuclei and bacterial DNA (small, grouped dots) are labeled with DAPI; anti-ezrin antibodies stained ezrin. Bar: 10 µm.

**c** Dose-dependent inhibition of signaling by WGA and Mal-I. **d** Inhibition of β-arrestin2-GFP recruitment under bacterial colonies by the selective inhibition of sialyltransferases with 3Fax-Peracetyl Neu5Ac (3FP-Neu5Ac). **e** Representative pictures of the dose–response experiment in **d**, bar: 10 µm, ***p < 0.001, n = 3.

**f, g** Inhibition of ezrin recruitment under colonies by 3FP-Neu5Ac. **f** Representative pictures of the dose–response experiment in **g**, bar: 10 µm, ***p < 0.001, n = 3 (ANOVA).
their activation. To address this hypothesis, in a dedicated experimental protocol designed to minimize the exogenous forces applied on bacteria (see Methods), wt and mutant meningococci deficient in PilT activity (ΔT30) were compared for their capacity to induce signaling in hCMEC/D3 cells. Supporting the contribution of pilus retraction in receptor activation, ezrin recruitment was significantly impaired under ΔT meningococci compared to wt under basal, static conditions (Fig. 6a, b).
under static conditions and after orbital agitation. Proterenol pre-treatment inhibited meningococcus signaling both pilus retraction and exogenous forces applied to bacteria. Iso-
maximal signaling depends on the additive effect of PilT-induced effect was signifi-
cantly larger for wt colonies, indicating that
absence of bacteria, can induce
β2AR signaling. ***P < 0.001 (ANOVA, n = 3 in duplicate). Representative images are shown in the left panel, bar: 10 µm

**Fig. 5 Terminal Neu5Ac contributes to meningococcus species specificity. a** A CMAH-KO clone, in which endogenous CMAH was mutated on both alleles (causing reading frame shift) using a CRISPR/Cas9 approach (Supplementary Fig. 8), was derived from C166 mouse endothelial cells. Wild-type or CMAH-
KO C166 cells expressing exogenous YFP-tagged human β2AR and CEACAM-1 (to induce mock bacterial adhesion) were infected with piliated non-
capsulated Opa+ (2C43 SiaD- Opa+) meningococci. Receptor distribution was examined by fluorescence microscopy. Receptor accumulated under bacterial colonies both in wt and ΔT meningococci. However, the effect was significantly larger for wt colonies, indicating that maximal signaling depends on the additive effect of PilT-induced pilus retraction and exogenous forces applied to bacteria. Iso-
proterenol pre-treatment inhibited meningococcus signaling both under static conditions and after orbital agitation.

The potential mechano-transducing role of the β2AR revealed in context of meningococcal infection was further investigated using Neu5Ac-selective WGA-coated beads in a reconstituted cell system. Beads were added to HEK-293 cell monolayers for 1 h, to promote WGA binding to Neu5Ac, and then submitted to orbital rotation. In the absence of rotation, only basal translocation of ezrin and β2AR was observed under the beads lying on cells expressing exogenous β2AR and myc-tagged β-arrestins. Rotation appliance significantly enhanced ezrin accumulation, but no accumulation was obtained in cells expressing the AT1R-YFP as receptor, or in cells expressing β2AR alone (Fig. 7). Thus, traction forces specifically applied on the β2AR via its glycans, even in the absence of bacteria, can induce β-arrestin-selective activation and downstream ezrin recruitment. In contrast to bacterial-mediated activation, however, the requirement for tandem glycan chains at a specific distance was not observed for the activation via WGA-coated beads. Indeed, wt β2AR or β2AR mutants lacking either N-linked glycan chain (N6A or N15A) were activated to a similar extent.

**Discussion**

From our data it appears that the activation of the β2AR by meningococcal Tfp involves traction forces applied via the bacterial pili on glycan chains located in the N-terminal extracellular region of the receptor. Part of the forces is generated intrinsically by the bacterial PilT ATPase, which controls Tfp retraction, extrinsic shear stress forces created by blood flow, and applied on bacteria providing the additional mechanical signal. Although previous studies reported that host–pathogen interactions can involve host cell glycans and that signaling properties of some receptors can be modulated by glycosylation, the GPCR activation mechanism reported here is unique. It involves NANA terminations two glycan chains located at a specific distance in the receptor N-terminus, independently of the protein backbone. Interestingly, the amino-acid residues mediating the activation of β2AR signaling have been identified for the major pilus component PilE. The PilE C-terminus domain, which contains a disulfide-bonded region (D-region), was found to be critical for inducing activation of the host cell response. In particular, a hyper-variable region confers specificity for signaling in endothelial cells via the β2AR. Among the four residues that are critical for signaling, two are positively charged lysine residues, an observation that fits well with the fact that this region would interact with the negatively charged NANA terminations the β2AR glycan chains. The particular distance of the receptor glycan chains required for bacterial-induced signaling activation probably reflects structural constraints, such as pilin orientation or...
spacings within the bacterial pilus. Supporting this hypothesis is the cooperativity that exists between the pilin subunits for full receptor activation (Supplementary Fig. 9).

The \(\beta_2\)AR is not the only GPCR displaying two glycan chains in its N-terminus at the “meningococcus infection-permissive” distance of 8–9 residues, since this feature is found in roughly 10% of GPCRs. However, only few of them are expressed in endothelial cells (i.e., bradikynin B2R, angiotensin AT2R, acetylcholine M3R) and they are not necessarily pre-associated with the adhesion receptor CD147 as the \(\beta_2\)AR, which is a key parameter in terms of meningococcal pathophysiology. Possibly because of the limited number of receptors that are potentially activated by pili, the recruitment of ezrin in response to mechanically stimulated \(\beta_2\)AR might be restricted to cell types expressing high concentrations of \(\beta\)-arrestins, such as endothelial cells. Indeed, a robust recruitment of ezrin under meningococcal colonies, and to WGA-coated beads in the HEK-293-reconstituted cell system, required the expression of exogenous \(\beta\)-arrestin, while these cells contain endogenous \(\beta\)-arrestins. Although \(\beta_2\)AR is accountable for most of the meningococcus-induced signaling in endothelial cells, a residual 10–15% ezrin recruitment under bacterial colonies was still observed upon receptor KO in hCMEC/D3 cells, consistent with the existence of some minor \(\beta_2\)AR-independent signaling.

Recent structural and molecular studies have markedly expanded our comprehension of the molecular mechanisms, which control GPCR function. For class A GPCRs (the class to which the \(\beta_2\)AR belongs) that are activated by non-peptide ligands, the orthosteric binding site, through which endogenous agonists activate the GPCR, is generally located in the middle of the seven-transmembrane helical bundle, between the extracellular loops and the middle plane of the plasma membrane. Allosteric ligands modulate receptor binding and activity by binding to sites, distinct from the orthosteric site, which can be located in variable positions. The binding site for a “small-molecule” allosteric modulator of the \(\beta_2\)AR was localized, for
The latter lectin has specificity for α-linked N-acetylgalactosamine. They were then let to sediment for 1 h on HEK-293 cells expressing the indicated receptors in the context of myogenic vasoconstriction. However, reminiscent of our findings, it was also reported that mechanical stretch can induce β-arrestin-selective signaling downstream of AT1Rs, the conformational change in β-arrestin induced by the stretch being similar to that induced by a β-arrestin-biased ligand. The mechano-transducing properties of the β2AR glycans and subsequent Tfp-mediated mechanical activation of the receptor might induce some signaling in the context of cell-to-cell interactions and/or diapedesis.

*Neisseria meningitidis* is a strict human pathogen. In addition to factors, which contribute to the survival of bacteria in the blood, such as transferrin binding or resistance to the complement, our data indicate that the mechanism of meningococcus-induced signaling itself contributes to species selectivity. Indeed, the presence of a single additional oxygen atom in Neu5Gc, the predominant mammalian form of NANA, compared to Neu5Ac, the predominant human form, is sufficient to impede pili binding to β2AR glycans and subsequent Tfp-mediated mechanical activation of the receptor. Our data suggest that additional factors...
might also contribute to meningococcal selectivity for its host. Indeed, mouse endothelial cell expressing Neu5Ac following inactivation of CMAH showed impaired initial adhesion. This observation indicates that the adhesion receptor (CD147 in humans) might also contribute to pathogen selectivity.

The Alu-mediated loss of CMAH about 2–3 million years ago, which determines the absence of Neu5Ac in humans and its replacement by Neu5Gc in the glycosalyx was probably maintained in humans because of some selective advantage, such as the resistance to an ancestral Neu5Gc-binding pathogen related to P. reichenowi, which infects non-human primates. Nevertheless, this early adaptation caused several pathophysiological consequences in humans, in addition to the susceptibility to meningococcal infection reported here. For example, the major erythrocyte-binding antigen-175 of Plasmodium falciparum, the parasite causing the most severe forms of human malaria, apparently evolved to take selective advantage of the excess of the Neu5Ac on human erythrocytes. Also, Salmonella typhi, an exclusive human pathogen that causes typhoid fever, produces a pathogenic toxin. This toxin binds to and is toxic for cells expressing glycans terminated in Neu5Ac over glycans terminated in Neu5Gc.

In conclusion, the β2-AR activation mechanism by meningococci, the specificity of this pathogen for humans, and the tether for meningococcal Tfp anchoring to endothelial cells actually implicate a common molecular element, Neu5Ac, a NANA form that is only found in humans. Traction forces applied via Tfp on Neu5Gc-terminated glycans of the pathogenic toxin. This toxin binds to and is toxic for cells expressing glycans terminated in Neu5Ac over glycans terminated in Neu5Gc.

Methods

Cell lines. The hCMEC/D3 human cell line, which retains major features of primary brain endothelial cells and express endogenous β-arrestins, β2-AR, and CEACAM-1, was described previously and is a kind gift from Pierre-Olivier Couraud, Institut Cochin, Paris, France. hCMEC/D3 cells were grown at a density of 25 × 10^4 cells per cm^2 in flask coated with 5 μg/cm^2 rat tail collagen type I (BD Bioscience), in EBM-2 medium (Lonza) supplemented with 5% of fetal calf serum (FCS), 1% of penicillin–streptomycin, 1.4 μM hydrocortisone, 5 μg/ml ascorbic acid, 10 mM HEPES, and 1 ng/ml basic fibroblast growth factor at 37 °C in a humid atmosphere in a 5% CO_2 incubator. HEK-293 (ATCC CRL-1573) cells were grown in flask coated with 0.01% poly-l-lysine (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS and 1% penicillin–streptomycin in 3% CO_2-enriched humidified atmosphere.

The mouse endothelial cell line C166 (ATCC CRL-2581), which express endogenous β2-AR, was grown in DMEM supplemented with 10% FCS and 1% penicillin–streptomycin in 5% CO_2-enriched humidified atmosphere.

The BHK cell line (ATCC PTA-3544) was grown in DMEM supplemented with 10% FCS and 1% penicillin–streptomycin in 5% CO_2-enriched humidified atmosphere.

Bacterial strains. Serogroup C meningococcal strain 8031, designated as 2C43, the non-encapsulated Opa expressing derivative, designated as 2C43ΔOp, and a mutant deficient in PilT activity derivative designated ΔT were used as specified in the Methods section (own collection). Bacteria were grown on Gonococcal Broth (GCB) agar plate with Kellogg’s supplement and the appropriate antibiotics. Kanamycin was used at 100 μg/ml and chloramphenicol was used at 6 μg/ml. Other strains have been used either variant of PilE plasmid (namely, PIIESA and PilESA variants or deleted of pilE gene (APIV) in the context of PilESA or PilESB variants, or deleted of pilE gene (APIV)Δ1830).

Treatment of cells and infections. On the day of infection, bacteria grown from an overnight culture on GCB agar plate were adjusted to OD_600 = 0.05 and incubated for 2 h at 37 °C in pre-warmed cell culture medium. Bacteria were added to cells at a multiplicity of infection of 100 and allowed to adhere for 30 min. Unbound bacteria were washed with fresh medium and infection was allowed to proceed for 2 h.

HCMEC/D3 cells were infected with wt 2C43 strain or its ΔT derivative (Figs. 3, 5, and 6). HEK-293 and C166 cells were infected with the non-encapsulated Saad-2C43ΔOp strain and its derivatives APIV, APIE and PIISA were used as controls.

Lectins used in infection-inhibition experiments were purchased from Vector laboratories (the complete list is shown in Supplementary Table 1 and used at a final concentration of 20 μg/ml, as recommended by the manufacturer). They were added 1 h before infection and again after 30 min of infection when fresh medium was added. Whereas MAL-ΔI did not inhibit bacterial adhesion to host cells, WGA partially inhibited bacterial adhesion at the used concentration. Since we showed previously that signaling was independent of adhesion, ezrin recruitment was examined below adherent colonies.

To quantify the role of pilus retraction and/or that of external forces like shear stress on ezrin recruitment, we set up a particular protocol of infection. To achieve stringent static conditions, cells were not washed after the 2 h infection and 16% paraformaldehyde (PFA) was added gently, directly to the infection medium (final PFA concentration: 4%). To apply continuous centrifugal forces mimicking shear stress, cells were placed on an orbital shaker at 100 r.p.m. Fixation was performed by adding 16% PFA on top of the cells.

Potassium depletion experiments were performed as described. Briefly, cells were rinsed with K^-free buffer (140 mM NaCl, 20 mM HEPES, 1 mM CaCl_2, 1 mM MgCl_2, 1 μM μ-g-glucose, pH 7.4) and then incubated in hypotonic buffer (K^-free buffer supplemented 1:1 with distilled water) for 5 min. Cells were then washed three times in the K^-free buffer and incubated for 20 min at 37 °C in the same buffer. In order to maintain proper bacterial growth during the potassium depletion assay, cell culture medium was added to K^-free buffer (1:5) during meningococcal infection.

Flow experiments. Cells were grown on disposable flow chambers (Slide VI, IBIDI) coated with 5 μg/cm^2 rat tail collagen type I. In total, 10^5 bacteria were added on top of the cells and allowed to adhere for 15 min without flow. Subsequently, a flow corresponding to a shear stress of 0.4 dyn/cm^2 was applied using a syringe pump (Harvard Apparatus). To quantify the surface of colonization, cells and bacteria were fixed using 4% PFA during 20 min; bacteria were then stained using anti-2C43 rabbit antibodies (1:1000, generously provided by Dr. P. Mangeat CNRS, UMR8539, Montpellier, France) and cells were stained using phalloidin. Bacteria were analyzed using an Incucyte S3 analysis system (Sartorius). Four images were acquired per IBIDI channel (~20 magnification). Surface of colonizing bacteria was determined using the Incucyte S3 software and expressed as median of colonization index with interquartile range (total surface of bacteria in μm^2 over total surface occupied by cells in μm^2).

Immunofluorescence on Mm-infected cells. After infection, cells were washed three times and fixed for 20 min in PBS containing 4% PFA and permeabilized 5 μg/ml saponin (PBS containing 0.1% BSA for 1 h). Cells were washed 3 times in PBS containing 0.1% bovine serum albumin (BSA) for 30 min and then with polyclonal anti-ezrin antibody (1:1000) in PBS containing 0.1% BSA for 1 h. After three washings in the same buffer, DIAP1 (4',6-diamidino-2-phenylindole; 0.5 μg/ml) was added to Alexa-conjugated secondary antibodies for 1 h (A-11003 and A-11035, Thermo Fisher Scientific, 1:200). After additional washings, coverslips were mounted in Mowiol. Image acquisition was performed on a laser-scanning confocal microscope (Leica TCS SP5). Images were collected and processed using the Leica Application Suite AF Lite (Leica) software.

Expression and purification of pilin recombinant proteins. Recombinant His-tag pilin fusion proteins were produced as periplasm-directed proteins as described before. Fragments of pilE, pilV, and pilM genes lacking the region coding for the amino-acid residues 1 to 28 of the full-length proteins were amplified by PCR from the existing plasmids pM-PilE, pM-PilV, and pM-PilM and subcloned in pET22B (Novagen) between BamHI and XhoI restriction sites. The primer sequences used are provided in Supplementary Table 2.

The pET22B plasmid carries a signal peptide to direct the recombinant protein into Escherichia coli periplasm, where disulphide bond formation can occur. These plasmids were transformed in the E. coli BL21 (DE3) (Thermo Fisher Scientific) strain and grown at 30 °C for 3 h in lysogeny broth (LB) and 16 h at 16 °C in LB + 1 mM isopropyl β-D-thiogalactoside (IPTG). The fusion proteins were extracted from the periplasm and loaded onto a Ni-NTA column (Thermo Scientific). Bound His-pilin were eluted using elution buffer (50 mM NaH_2PO_4, pH 8.3, 300 mM NaCl, 250 mM imidazole) and then dialyzed using an Amicon 10k device and re-dissolved in PBS at a concentration of 1 mg/ml. Purity and quality of recombinant proteins were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.
Plasmins and transfections. hCMEC/D3 and C166 cells were transfected using the Amaxa Nucleofactor Kit for HUVEC (Amaza Biosystem), according to the manufacturer’s instructions, with 2 μg of plasmids per 106 cells. HEK-293 cells were transfected using 1 μL of Lipofectamine 2000 (Invitrogen) and 0.1 μg of each plasmid per 100 μL of final mix. BHK cells were transfected using Genejuice Transfection Reagent (Novagen) according to the manufacturer’s instructions. Cells (1 × 105) were transfected with plasmid DNA containing HA-tag, TMβ1AR-YFP (5 μg) and processed with HTTR for 48 h later. The plasmid encoding HA-ECl-TMβ1AR-YFP was constructed using a two-step procedure. First, the YFP cDNA (pEYFP-N1 vector; Clontech) was amplified by PCR using primers containing BsrEI site. The PCR product was then subcloned in phase downstream of the 3′acceptor for the HA-βAR-YFP plasmid23 using the BsrEI site. The plasmid was sequenced to verify the phase and the absence of undesired mutations (DNA sequence facility, Institut Cochin).

Construction of plasmids for cmyc-bar1/2 and GFP-bar35, the human βAR fused to YFP (βAR-YFP)34, the AT1R29 were described previously. The plasmid encoding CECAM1-19 was kindly provided by Dr. Muntaz Virji (Department of Cellular and Molecular Medicine, School of Medical Sciences, University of Bristol, Bristol, UK). The plasmid encoding the mouse βAR fused to YFP (βAR-YFP) was obtained by PCR amplification from mouse βAR cDNA and subsequent sub-cloning of the PCR product into the pEYFP-N1 plasmid (Clontech) within Xhol and HindIII restriction sites. The primer sequences used (βAR_Fw and βAR_Rv) are provided in Supplementary Table 2. The plasmid coding for βAR-N6A-YFP, βAR-N15A-YFP, HA-AT1R-N1, HA-2AR-N6A, and HA-2AR-N15A constructs were obtained by site-directed mutagenesis (QuickChange®; Stratagene). Homogeneous time-resolved FRET. HTRF measures energy transfer of between cryptate donors (610HATAA, monoclonal antibody (Mab) anti HA-Tb cryptate, Cisbio) and acceptor fluorescence acceptor molecule (d2, Cisbio, Inc.) 

βAR-N6A constructs were obtained by site-directed mutagenesis with a ×100 objective, a ultraviolet microscope equipped with an x100 objective, a 488-nm short-pass filter and a 546-nm long-pass filter. For each field, the number of colonies was counted and the presence or absence of the protein of interest below colonies was determined. The focal plane was adjusted just above the apical membrane. Recruitment of the protein of interest was counted as positive if: (i) the recruited protein was accumulated below colonies in a ring shape around the bacteria. This shape is also called honeycomb shape, and (ii) if the fluorescence intensity was greater than the negative control intensity on the plasma membrane. At least 50 colonies were observed per coverslip. Each experiment was repeated several times in triplicate. Data were examined for significance using the Prism GraphPad software.

Quantification of ezrin recruitment below colonies was also performed by measuring the intensity of fluorescence signals (as in Fig. 6b): 10 fields per slides were acquired with a Zeiss Apotome 2.0. Each colony was analyzed using the ImageJ software. Briefly, the DAPI layer and ezrin staining layer were extracted and a threshold mask was applied on the ezrin staining layer to suppress the background staining (normal ezrin staining at the plasma membrane) and to transform the layer into a black and white image. The mean value of pixel intensity obtained below colonies covered by ezrin staining and is expressed as Arbitrary Intensity of ezrin recruitment.

Trypsin shaving and immunoprecipitation of βAR. HEK-239 cells were transfected with the indicated plasmids and cultivated for 36 h. The cells were harvested in PBS-EDTA (0.5 μM EDTA in PBS) and digested with trypsin (1 μg/ml) (Pro-mega, catalog #5111A) according to the manufacturer’s protocol at 37 °C for 1 h. The trypsin digestion yields the pellets using 100 μL of 0.5 μM HEPES, pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, and complete protease inhibitor (Roche) on ice for 30 min. Cell lysates were then centrifuged at 13,000 rpm for 15 min at 4 °C. Supernatants were transferred to a fresh tube and the presence of cleaved receptor N-termini was verified by western blot. Proteins were separated on SDS-PAGE on a 10% (w/v) polyacrylamide resolving gel. The proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in 0.1% TBST (Tris-buffered saline containing 0.1% Tween-20 (Sigma)) for 1 h at RT. The membranes were incubated with primary antibodies anti-βAR (Cell Signaling) (1:1000) and anti-vinculin (1:5000) (Sigma) overnight at 4 °C. The following day, the membranes are washed three times with 0.1% BSA in PBS and washed with 0.1% BSA in PBS 3 times with IRDye® 800 CW Donkey anti-Rabbit and IRDye® 680 CW Donkey anti-Mouse (LI-COR®) (1:10,000). The blots were washed two times with 0.1% TBST and one time with PBS and developed using Odyssey® CLx (LI-COR).

MS analysis of HA-purified peptides. Trypsin-cleaved N-termini of wt and mutant βAR bound to HA beads were submitted to On-Bead PNGase digestion (0.5 μL, Fast PNGase F, New England Biolabs) in a final volume of 15 μL of PNGase F digestion buffer (50 mM NaH2PO4, pH 7.4, 10% glycerol and reducing buffer for 10 min at 50 °C on a thermomix (Eppendorf). Supernatants were then collected and diluted to a 50 μl final volume with 50 mM (NH4)2CO3 and media 48 h after transfection. Cells were then seeded at decreasing densities in 6-well plates. Clones were selected after 2 weeks and amplified before selection. KO clones were selected as described above.
β-Sialic acid detection assay. Heating the sample with 2 M acetic acid at 80 °C for 3 h, followed by the addition of 100 μL of 2 M NaOH to neutralize the solution. The standard curve and the concentration of the NANA quantitation were determined according to the manufacturer’s protocol. To normalize the results according to the actual amount of fragments immunoprecipitated with HA beads, 10% of the beads were used instead of the whole beads and eluted with 2 x loading dye and analyzed by western blot. The specific anti-HA labeled material was quantified using Image Studio Lite (LI-COR).

Sialic acid detection assay. NANA quantification on immunoprecipitated wt and mutant βAR N-termini after trypsin digestion was performed using the NANA Colorimetric/Fluorometric Assay Kit (Bio Vision Incorporated, Catalog #K566-100). Free NANA was released from the HA-bead immunoprecipitated material by heating the sample with 2 M acetic acid at 80 °C for 3 h, followed by the addition of 100 μL of 2 M NaOH to neutralize the solution. The standard curve and the concentration of the NANA for the different samples were determined according to the manufacturer’s protocol. To normalize the results according to the actual amount of fragments immunoprecipitated with HA beads, 10% of the beads were used instead of the whole beads and eluted with 2 x loading dye and analyzed by western blot. The specific anti-HA labeled material was quantified using Image Studio Lite (LI-COR).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request. A reporting summary for this article is available as a Supplementary Information file.

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