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Neisseria gonorrhoeae Blocks Epithelial Exfoliation by Nitric-Oxide-Mediated Metabolic Cross Talk to Promote Colonization in Mice

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SUMMARY

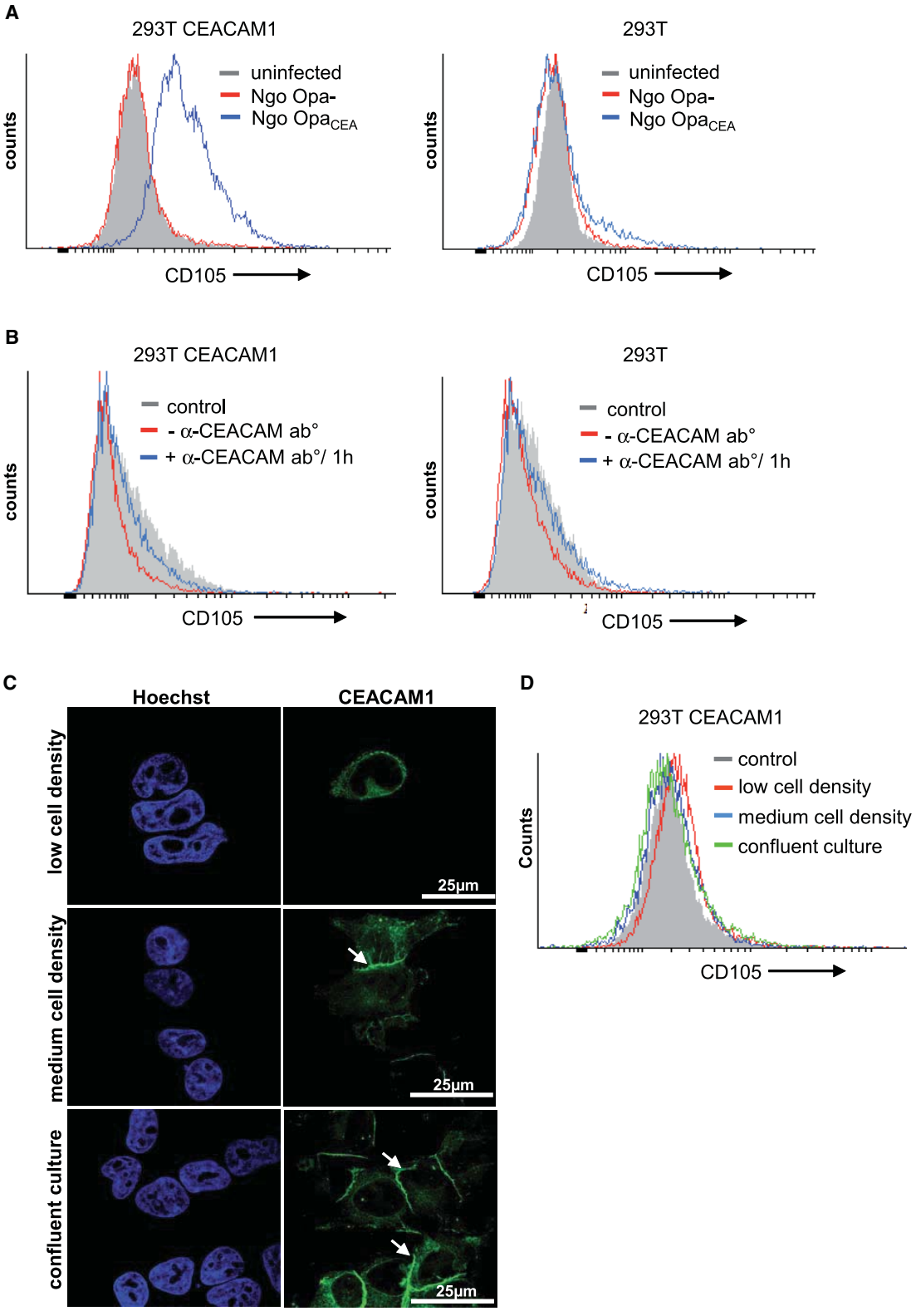
Several pathogens suppress exfoliation, a key defense of epithelia against microbial colonization. Common among these pathogens, exemplified by *Neisseria gonorrhoeae*, is their ability to bind carcinoembryonic antigen-related cell adhesion molecules (CEACAMs). Gonococcal CEACAM engagement triggers the expression of CD105, which is necessary to block epithelial exfoliation, whereas homotypic CEACAM-CEACAM interactions or antibody-mediated CEACAM clustering does not lead to CD105 expression. Here, we show that CEACAM-associated bacteria release nitric oxide (NO) during anaerobic respiration, and membrane-permeable NO initiates a eukaryotic signaling pathway involving soluble guanylate cyclase (sGC), protein kinase G, and the transcription factor CREB to upregulate CD105 expression. A murine vaginal infection model with *N. gonorrhoeae* reveals this metabolic cross communication allows bacterial suppression of epithelial exfoliation to facilitate mucosal colonization. Disrupting NO-initiated responses in host cells re-establishes epithelial exfoliation and inhibits mouse genital tract colonization by *N. gonorrhoeae*, suggesting a host-directed approach to prevent bacterial infections.

INTRODUCTION

Neisseria gonorrhoeae is a human-restricted bacterial pathogen, which causes one of the most common sexually transmitted diseases (Handsfield, 1990). Gonococci constitute a global health threat as vaccination is not available and multi-drug resistant isolates are on the rise (Wi et al., 2017).

Similar to other human-restricted bacteria, such as *Neisseria meningitidis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Fusobacterium spec*, *Moraxella catarrhalis*, or human-pathogenic isolates of *Escherichia coli*, gonococci exploit a family of epithelial membrane receptors for host colonization (Brewer et al., 2019; Hill and Virji, 2003; Königer et al., 2016; Tchoupa et al., 2014; Virji et al., 1996). These receptors belong to the group of carcinoembryonic antigen (CEA)-related cell adhesion molecules (CEACAMs), which can be found on mucosal surfaces throughout the human body (Gray-Owen and Blumberg, 2006; Kuespert et al., 2006). In particular, CEACAM1, CEA (the product of the *CEACAM5* gene), and CEACAM6 are expressed on the apical membrane of various epithelial cells and can serve as an initial anchor for the incoming CEACAM-binding pathogens. To connect to host CEACAMs, microbes employ distinct, structurally unrelated adhesins, such as the outer membrane embedded Opa protein adhesins of *N. gonorrhoeae* (Chen et al., 1997), the coiled-coil stalk of the UspA1 protein of *M. catarrhalis* (Hill and Virji, 2003), the beta-barrel protein OMP P1 of *H. influenzae* (Tchoupa et al., 2015), or the autotransporter HopQ protein of *H. pylori* (Königer et al., 2016). The independent evolution of various CEACAM-binding proteins implies that this trait provides a significant advantage for microbes colonizing the human mucosa. Clearly, intimate association with the epithelial surface can benefit the initial establishment of pathogens in their host. However, CEACAM binding by bacteria promotes mucosal colonization beyond mere physical interaction (Gray-Owen, 2003; Tchoupa et al., 2014). In the case of *Neisseria gonorrhoeae*, it has been demonstrated that CEACAM engagement by the bacteria results in altered gene expression including *de novo* expression of CD105 (also known as endoglin), a protein usually not found on epithelial cells and rather expressed by endothelial cells (Muenzner et al., 2005). CD105 expression is necessary and sufficient for a phenotypic change of the infected cells, which show increased integrin activity and enhanced binding to extracellular matrix proteins (Muenzner et al., 2005). In the vaginal and cervical epithelium of the female genital tract, where in several regions superficial epithelial cells are readily shed in response to bacterial infection (Evans, 1977; McGee et al., 1981; Melly et al., 1981; Wang et al., 2017; Yu et al., 2019), the enhanced integrin-mediated matrix attachment of the infected cells translates into reduced exfoliation (Muenzner et al., 2010, 2016). Together, the intimate association of the microbes with CEACAMs on the cell surface and the resulting suppression of exfoliation facilitate establishment of pathogens in their host by providing a stable platform for mucosal colonization (Hauck et al., 2012).

Though CEACAM family members have been implicated in modulating cellular signaling events (Huber et al., 1999; Leung



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et al., 2008; Nagaishi et al., 2006; Poy et al., 2002), how CEACAM clustering at the plasma membrane leads to altered gene expression in the nucleus is unclear. The ability of CEACAMs to modulate gene expression appears particularly intriguing as several family members, such as CEA or CEACAM6, which can induce CD105 expression in response to gonococcal binding, are GPI-anchored proteins (Muenzner et al., 2005). Furthermore, a CEACAM1 variant with a complete deletion of the cytoplasmatic domain has also been shown to trigger CD105 expression upon engagement by Opa protein expressing gonococci (Muenzner et al., 2005). We speculated that CEACAM clustering by multivalent bacteria could result in the recruitment of a putative co-receptor, which would then mediate downstream responses including CD105 expression.

Here, we report that CEACAM clustering in the absence of bacteria or CEACAM engagement by metabolically inactive bacteria does not lead to downstream signaling. Rather, CD105 expression is initiated by a membrane diffusible gas, nitric oxide (NO), which is produced by gonococci during anaerobic metabolism. This unforeseen metabolic cross communication between host-cell-associated bacteria and NO-responsive signaling modules in mammals allow gonococci to enhance host cell-matrix adhesion, to suppress exfoliation, and to facilitate mucosal colonization. Bacterial NO release not only represents an unexpected communication channel between intimately host-associated microbes and the epithelium but also opens an unexplored route to prevent the initial establishment of bacterial pathogens on the human mucosa.

RESULTS

CEACAM Engagement by Bacteria, but Not Homotypic CEACAM Interaction or Antibody-Mediated Clustering, Leads to CD105 Expression

In previous studies, we have demonstrated that engagement of epithelial CEACAMs, such as CEACAM1, CEA, or CEACAM6 by CEACAM-binding strains of *Neisseria gonorrhoeae* or of *E. coli* leads to the upregulation of CD105 (Muenzner et al., 2010, 2016). Indeed, when human embryonic kidney 293 (293T) cells stably expressing CEACAM1ΔCT-GFP (293T CEACAM1; Figure S1) were infected with Opa_{CEA}-expressing *Neisseria gonorrhoeae* for 8 h, the infected cells showed surface expression of CD105 (Figure 1A). In contrast, infection of 293T CEACAM1 cells with an isogenic, non-opaque gonococcal strain did not result in CD105 expression (Figure 1A). Furthermore, when the parent 293T cells, which do not express any CEACAM family member, were infected with either the CEACAM-binding or the non-CEACAM-binding gonococcal strain, no alteration in CD105 expression was observed (Figure 1A). These data support the view that CEACAM engagement by CEACAM-binding bacteria is necessary for the *de novo* expression of CD105 in epithelial cells. Surprisingly, when CEACAM1 was stimulated by a monoclonal CEACAM1-binding antibody, which bound with high specificity to the 293T CEACAM1 cells and was further cross-linked with a polyclonal anti-mouse antibody to cluster CEACAM1, no change in CD105 expression was observed (Figures 1B and S1). Furthermore, when 293T CEACAM1 cells were grown at increasing cell densities, which promotes physiological CEACAM1-CEACAM1 interactions (Figure 1C), again no CD105 expression was observed (Figure 1D). Together, these results suggest that CEACAM engagement or clustering is not sufficient to trigger upregulation of CD105 and that bacterial CEACAM engagement differs from physiological CEACAM clustering.

A PKG-Dependent Signaling Cascade Induces CD105 Expression

We speculated that cell-associated bacteria might initiate, in parallel to CEACAM clustering, additional host signaling pathways, e.g., by stimulating potential CEACAM1 co-receptors. To get an idea of the intracellular signaling pathways instrumental for triggering CD105 expression, we employed a pharmacological approach. Using a panel of protein kinase inhibitors, we observed that a protein kinase G inhibitor (PKGi), but not inhibitors against PKA, PKC, or Ca^{2+}/Calmodulin-dependent kinase II (CamKII) completely abolished the bacteria-triggered upregulation of CD105 (Figures 2A and 2B). To verify the ability of PKG to stimulate CD105 expression, constitutive active (ca) forms of two PKG enzymes, PKG type I alpha (PKGα), and PKGI beta (PKGβ) were expressed in 293T cells (Figure S1). In the absence of bacterial infection, expression of either active PKGI isoform resulted in a gain of function with regard to CD105 expression, while overexpression of an unrelated protein tyrosine kinase (focal adhesion kinase; FAK) did not lead to alterations in CD105 levels (Figure 2C). PKG is embedded in a canonical signaling pathway, which senses

Figure 1. CEACAM Engagement by Bacteria, but Not Homotypic CEACAM Interaction or Antibody-Mediated Clustering, Leads to CD105 Expression

(A) 293T CEACAM1 cells (left panel) or 293T cells (right panel) were infected for 16 h with non-opaque, non-CEACAM-binding gonococci (Ngo Opa−), Opa_{CEA}-expressing, CEACAM-binding gonococci (Ngo Opa_{CEA}), or left uninfected (control). Cells were stained with a monoclonal α-CD105 antibody and AlexaFluor405-conjugated goat-α-mouse antibodies and analyzed by flow cytometry. Infection with CEACAM-binding bacteria (blue line) increased CD105 expression by 293T CEACAM1 cells, whereas infection with Ngo Opa− had no effect (red line). Infection of 293 cells did not lead to CD105 expression. Gray area indicates staining of uninfected cells.

(B) 293T CEACAM1 cells were incubated or not with 3 μg/mL monoclonal α-CEACAM antibody (clone D14HD11) for 1 h at 4°C, washed with PBS, and mouse antibodies were clustered for 1 h at 4°C with a polyclonal goat α-mouse antibody. Cells were then cultured over night at 37°C and analyzed for CD105 expression as in (A). Gray area indicates staining of cells with an isotype matched control antibody. Shown is one representative experiment out of three independent biological replicates.

(C) 293T CEACAM1 cells were seeded at low, medium, or high cell densities. Next day, cells were fixed and analyzed for CEACAM localization by staining with monoclonal α-CEACAM antibody (green), cell nuclei were visualized by hoechst (blue). Arrows indicate homotypic CEACAM interaction at cell-cell attachment sites.

(D) Cells were seeded as in (C) for 24 h and analyzed for CD105 expression by flow cytometry. Gray area indicates staining of cells with an isotype matched control antibody. Shown is one representative experiment out of three independent biological replicates.

See also Figure S1.

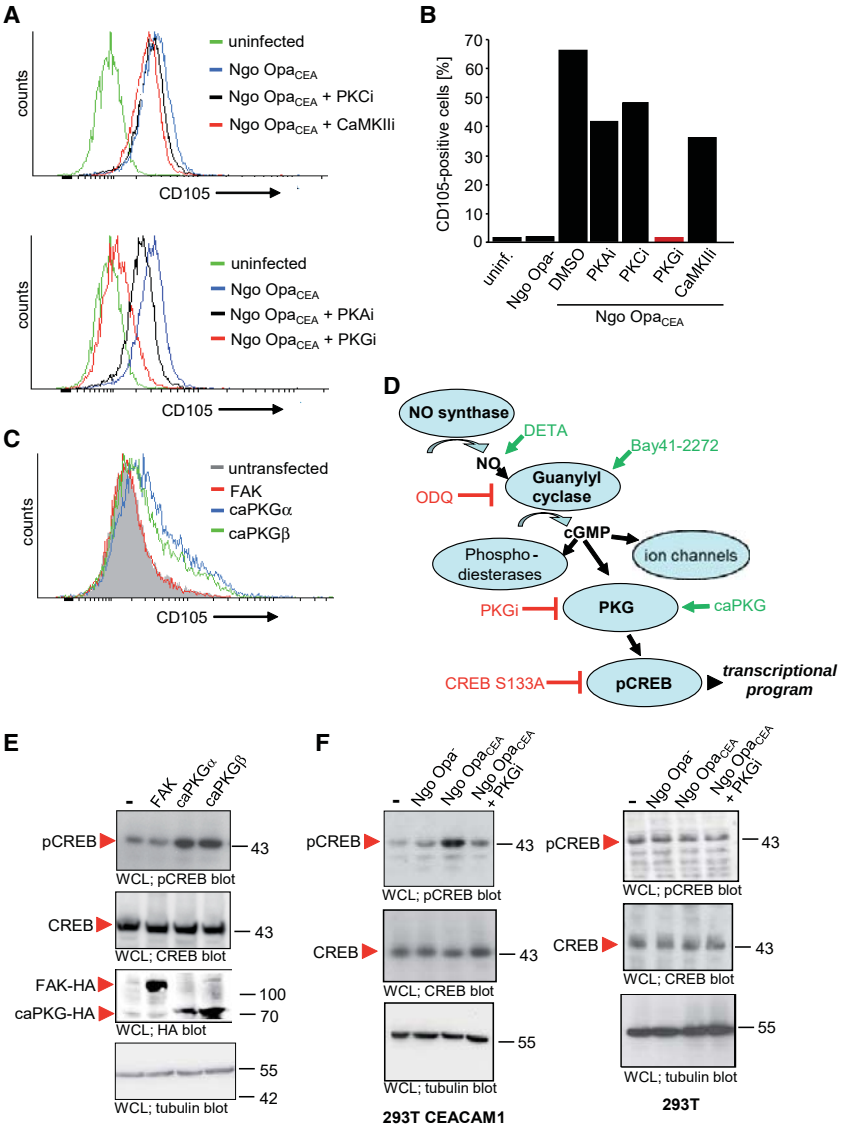

Figure 2. A PKG-Dependent Signaling Cascade Induces CD105 Expression

(A) 293T CEACAM1 cells were infected with CEACAM-binding, Opa$_{CEA}$-expressing (Ngo Opa$_{CEA}$) or non-opaque gonococci (Ngo Opa−), or left uninfected (control). In addition, cells received the inhibitors specific for protein kinase A (PKAi), PKCi, PKGi, Camodulin-dependent kinase II (CaMKIIi), or solvent (DMSO). 8 h after infection, cells were stained with a monoclonal α-CD105 antibody and AlexaFluor405-conjugated goat-anti-mouse antibodies and analyzed by flow cytometry. Shown are representative experiments.

(B) The bar graph shows the mean percentage of CD105-positive cells (10,000 analyzed events each) from three independent experiments executed as described in (A).

(C) 293T cells were transfected with plasmids encoding either constitutive active (ca) PKG Iα, caPKG Iβ or FAK, or left untransfected. 2 days later, cells were stained for CD105 and analyzed by flow cytometry.

(D) Scheme depicting the canonical NO-triggered signaling cascade in mammals. The used agonistic and antagonistic treatments are indicated in green and red, respectively.

(E) 293T CEACAM1 cells were transfected with plasmids encoding HA-tagged caPKGα, caPKGβ, or FAK, or left untransfected. 2 days later, whole cell lysates (WCLs) were probed with antibodies against phosphoCREB (pCREB), CREB, HA-tag, or tubulin.

(F) 293T CEACAM1 cells (left panels) or 293T cells (right panels) were infected as in (A) for 16 h. Indicated samples were treated with 5 μM PKG inhibitor (PKGi). WCLs were probed with antibodies against pCREB, CREB, or tubulin.

See also Figure S1.

elevated levels of the second messenger cyclic guanosine monophosphate (cGMP) and modulates the expression of target genes by phosphorylating and activating transcription factors of the CREB family (Figure 2D). Indeed, upregulation of CD105 expression by caPKGI isoforms correlated with elevated levels of phosphorylated CREB (pCREB) (Figure 2E). In line with the idea that PKG and CREB are involved in cellular responses to CEACAM-binding bacteria, infection of 293T CEACAM1 cells, but not wild-type 293T cells by Opa$_{CEA}$-expressing *N. gonorrhoeae* was accompanied by increased PKG-dependent phosphorylation of CREB (Figure 2F). Together, these findings suggest that PKG signaling is critical for the upregulation of CD105 expression by epithelial cells in response to bacterial engagement of CEACAMs.

NO Triggers PKG Signaling and Leads to CD105 Expression by Epithelial Cells

In eukaryotic cells, a rise in cGMP levels results from the activity of guanylate cyclases (GCs), which occur in the form of integral membrane proteins or cytosolic soluble enzymes (soluble GC; sGC) (Mónica et al., 2016). sGC, in turn, is activated by the radical NO, a short-range gaseous messenger molecule (Figure 2D). Importantly, a selective inhibitor of sGC (1H-[1,2, 4]oxadiazolo[4,3,-a]quinoxalin-1-one [ODQ]; Zhao et al., 2000) strongly reduced CREB phosphorylation (Figure 3A) and CD105 expression (Figure 3B) in response to bacterial CEACAM engagement, while ODQ did not affect bacterial viability (Figure S2). As reported previously (Muenzner et al., 2010, 2005), CD105 expression triggered by CEACAM-binding, Opa$_{CEA}$-expressing gonococci is linked to the reduced detachment of epithelial cells from a collagen matrix *in vitro*, while infection with non-opaque bacteria leads to cell detachment (Figure 3C). Importantly, the ability of Opa$_{CEA}$-expressing gonococci to suppress epithelial cell detachment was reverted by ODQ treatment (Figure 3C) indicating that sGC is required downstream of bacterial CEACAM engagement to trigger CD105 expression and enhance cell-matrix attachment. In line with this idea, an agonist of sGC (BAY 41–2272) triggered phosphorylation of CREB and promoted CD105 expression by epithelial cells independent of bacterial infection (Figures 3D and 3E). While the increased CREB phosphorylation upon bacterial infection was blocked

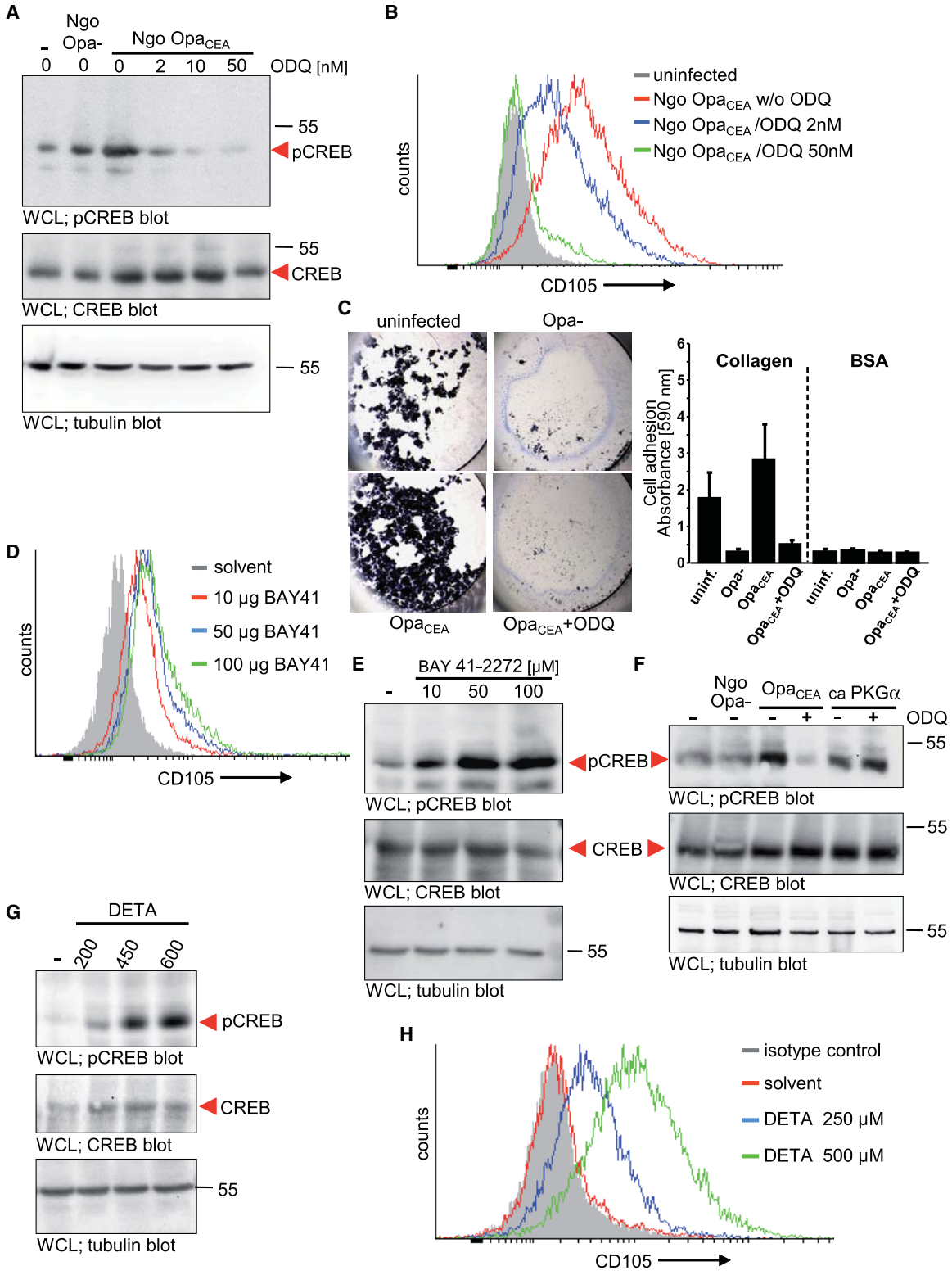

Figure 3. NO Triggers PKG Signaling and Leads to CD105 Expression by Epithelial Cells

(A) 293T CEACAM1 cells were infected CEACAM-binding, Opa$_{CEA}$-expressing (Ngo Opa$_{CEA}$), non-opaque gonococci (Ngo Opa−), or left uninfected (−). Cells received the indicated amounts of the sGC inhibitor ODQ or solvent. WCLs were analyzed by western blotting using antibodies against phosphoCREB (upper panel), total CREB (middle panel), or tubulin (lower panel).

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by ODQ, the sGC inhibitor did not interfere with CREB phosphorylation induced by caPKG (Figure 3F). These results suggest that sGC, PKG, and CREB act in a signaling pathway to increase CD105 expression and thereby promote host cell-matrix adhesion in response to bacterial CEACAM engagement. Similar to CEACAM-binding gonococci, CEACAM-binding *E. coli*, such as the AfaE-III adhesin-encoding strain A30, have also been shown to trigger CD105 expression (Muenzner et al., 2016). The CD105 expression induced by AfaE-III-expressing *E. coli*, but not by the adhesin-deficient isogenic strain, was paralled by a strong increase in CREB phosphorylation (Figure S3). Most importantly, CREB phosphorylation and CD105 expression triggered by *E. coli* AfaE-III were completely abrogated by pretreatment of 293T CEACAM1 cells with the sGC inhibitor ODQ or with the PKG inhibitor (Figure S3), suggesting that stimulation of sGC-PKG-CREB signaling leading to CD105 expression can occur in response to several CEACAM-binding bacteria.

As NO is a physiological stimulator of sGC, we wondered whether addition of NO would mimic the effect of bacterial infection on epithelial cells. Indeed, treatment of 293T cells with DETA NONOate (2,2′-(2-hydroxy-2-nitrosohydrazinylidene)bis-ethanamine), a chemical NO donor, triggered CREB phosphorylation and pronounced expression of CD105 (Figures 3G and 3H). Also in cell lines derived from gonococcal target tissues, such as immortalized human vaginal epithelial cells as well as ME180 cervix carcinoma cells, application of DETA or stimulation of sGC with BAY 41–2272 led to CD105 expression, while application of ODQ or a PKG inhibitor blocked the bacteria-triggered increase in CD105 expression (Figure S4). Together, these results reveal that concomitant with the bacterial engagement of CEACAMs, a NO-initiated signaling pathway is elicited in infected human epithelial cells, which is ultimately responsible for the upregulation of CD105.

CREB-Binding Sites in the CD105 Promoter Mediate the Transcriptional Response to Increased NO Levels

If enhanced expression of CD105 is due to an intracellular NO-triggered cascade, this should be reflected by NO-PKG-CREB-dependent transcriptional activity of the CD105 promoter. Indeed, a ~2 kb fragment of the human CD105 promoter contains two predicted CREB-binding sites (Figure S5A) (Graulich et al., 1999). In luciferase reporter assays, this fragment showed strong transcriptional activity upon infection of CEACAM-expressing cells with CEACAM-binding gonococci, while a promoterless control construct or a truncated CD105 promoter (~400 bp) led to weak luciferase expression under these conditions (Figure S5B). Importantly, deletion of the CREB-binding site ~700 bp upstream of the transcriptional start site (CRE-1) completely abolished the bacteria-induced activity of the 2-kb CD105 promoter fragment (Figure S5B). Furthermore, co-expression of a dominant-negative CREB mutant (CREB S133A) blunted the activity of the 2-kb CD105 promoter fragment upon bacterial infection (Figures S5B and S5C). Supporting the idea that the CD105 promoter strongly responds to NO-signaling, addition of the sGC agonist BAY 41–2272, DETA, or overexpression of caPKGα resulted in strong luciferase activity, which depended on the CRE1 site and occurred in these cases in the absence of bacterial infection (Figures S5D and S5E). Together, these results demonstrated that a host cell intrinsic, NO-triggered signaling pathway leads to CD105 promoter activity and that the identical components are instrumental to allow upregulation of CD105 expression by CEACAM-binding gonococci.

Anaerobic Nitrite Respiration by Bacteria Is the Main Source of NO

Under inflammatory conditions, inducible NO synthase (iNOS) can be expressed by multiple cell types, including epithelial cells. iNOS and other eukaryotic NOS enzymes convert L-arginine to L-citrulline thereby releasing NO (Alderton et al., 2001). To investigate, if the NO responsible for CD105 expression in epithelial cells might originate from the activity of endogenous NOS, we employed N^G,N^G-dimethyl-L-arginine (ADMA), a specific inhibitor of eukaryotic NOS enzymes. Surprisingly, pharmacological blockade of NOS activity did not reduce CREB phosphorylation or CD105 expression by CEACAM-expressing cells upon infection with CEACAM-binding gonococci (Figure 4A). Therefore, we considered the infecting microorganisms as an alternative source of NO, as this radical occurs as a metabolic intermediate in many bacteria (Poole, 2005; Zumft, 1997). Importantly, formaldehyde fixation of gonococci, which does not destroy their CEACAM-binding properties (Figure S6), completely abrogated their ability to induce CD105 expression in epithelial cells (Figure 4B). This finding indicated that only metabolically active bacteria can trigger this host cell response. One candidate metabolic reaction responsible for NO production by *N. gonorrhoeae* is the reduction of nitrite. Gonococci can employ a truncated denitrification pathway, which uses nitrite as an electron acceptor (Figure 4C). The nitrite reductase AniA catalyzes this reaction and releases NO, which can be further reduced to nitrous oxide N_2O by the NO reductase NorB (Figure 4C). Denitrification in

(B) 293T CEACAM1 cells were infected or not with Ngo Opa_{CEA} and treated with ODQ as indicated. Cells were analyzed for CD105 expression by flow cytometry. Gray area indicates staining of uninfected cells. Shown is a representative result of three experiments.
(C) 293T CEACAM1 cells were infected or not with Ngo Opa− or Ngo Opa_{CEA} for 16 h in the presence of ODQ as indicated. Cells were detached and plated on collagen or BSA for 90 min. Following washing, adherent cells were fixed stained with crystal violet. Left panels show representative field of views of cells plated on collagen. Crystal violet was eluted and the absorbance at 590 nm was measured. Bars represent mean ± SD from quadruplicate wells.
(D) 293T CEACAM1 cells were incubated with the indicated amounts of the sGC stimulator BAY 41–2272 or solvent. After 16 h, cells were analyzed by flow cytometry for CD105 expression. Gray area indicates staining of solvent-treated cells.
(E) 293T CEACAM1 cells were treated as in (D) and whole cell lysates were analyzed by western blotting as in (A).
(F) 293T CEACAM1 cells were transfected with caPKGα or were infected with Ngo Opa−, Ngo Opa_{CEA}, or left uninfected. Cells were treated with ODQ as indicated and WCLs were analyzed by western blotting as in (A).
(G) 293T CEACAM1 cells were treated for 6 h with the indicated concentrations of the NO-releasing compound DETA or solvent and WCLs were analyzed by western blotting as in (A).
(H) Samples as in (G) were analyzed by flow cytometry for CD105 expression. Gray area indicates staining of untreated cells with a control antibody.
See also Figures S2–S5.

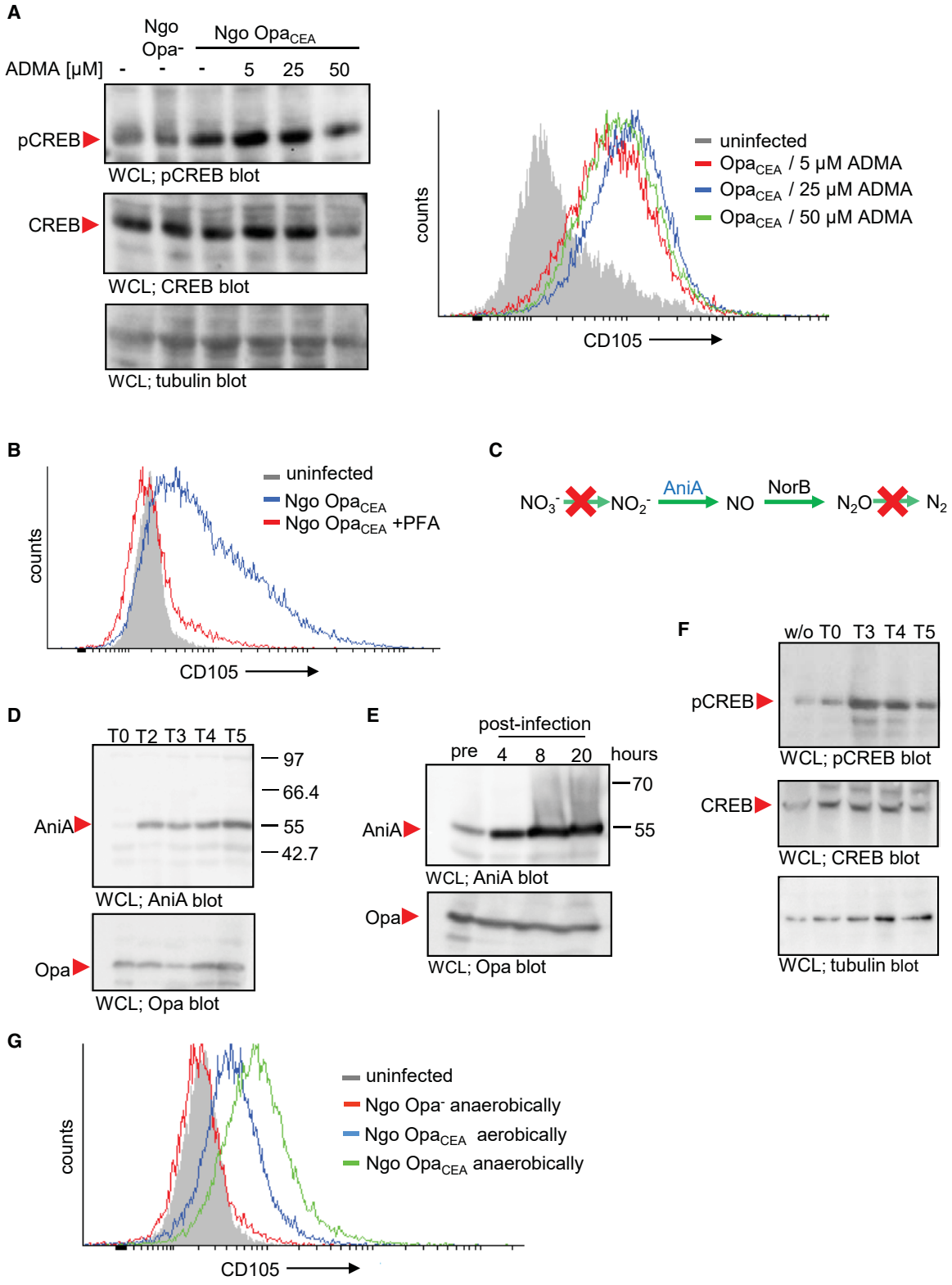

Figure 4. Anaerobic Nitrite Respiration by Gonococci Is the Main Source of NO

(A) 293T CEACAM1 cells were infected with non-CEACAM-binding (Ngo Opa−) or Opa$_{CEA}$-expressing, CEACAM-binding gonococci (Ngo Opa$_{CEA}$), or left uninfected. 30 min prior to the infection with Opa-expressing bacteria the cells were incubated with the indicated amounts of ADMA. Whole cell lysates were

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gonococci only occurs under anaerobic conditions, as AniA expression is tightly regulated by oxygen (Mellies et al., 1997). Thus, under standard growth conditions, AniA expression by *N. gonorrhoeae* was marginal (Figure 4D). However, upon anaerobic culture in the presence of nitrite, strong expression of AniA was observed (Figure 4D). We reasoned that AniA might also be upregulated in the course of *in vitro* infection assays, which are performed under static, non-aerated growth conditions. Indeed, within 4–8 h after infection of epithelial cells *in vitro*, AniA was strongly expressed by the gonococci (Figure 4E), indicating that oxygen levels in the infected cultures drop rapidly and induce anaerobic nitrite respiration by the bacteria. Most importantly, pre-conditioning of gonococci to anaerobic growth before infection resulted in elevated levels of CREB phosphorylation and a doubling of CD105 upregulation by the infected epithelial cells (Figures 4F and 4G). This increased expression of CD105 again depended on the ability of *N. gonorrhoeae* to tightly bind to the host cell surface via the CEACAM-binding Opa_{CEA}-adhesin, as Opa negative (Opa−) gonococci did not lead to enhanced CD105 expression, even when these bacteria were pre-conditioned to anaerobic growth (Figure 4G). Together, these results suggest that the anaerobic nitrite metabolism of gonococci is responsible for the stimulation of an NO-sensitive signaling pathway in the eukaryotic host cell. This metabolic cross communication requires intimate contact between the bacteria and the infected cells as the radical NO can only act within micrometer distance (Furchgott and Jothianandan, 1991).

Purified Gonococcal AniA Can Induce CD105 Expression and Suppresses Epithelial Exfoliation

Under anaerobic conditions, AniA is an essential protein for gonococci (Mellies et al., 1997). Accordingly, a genetic loss-of-function experiment with AniA mutant bacteria was not feasible. Therefore, we produced wild-type AniA and a nitrite reductase-deficient variant of AniA (AniA H280L) as recombinant proteins in *E. coli* (Figure S7). The purified wild-type enzyme generated NO from nitrite, while AniA H280L was inactive (Figure S7). Addition of wild-type AniA, but not AniA H280L, to epithelial cell cultures induced CREB phosphorylation and CD105 expression in the absence of bacterial infection (Figures 5A and 5B), demonstrating that exogenous AniA can produce sufficient amounts of NO to trigger the sGC-PKG-CREB intracellular signaling cascade in epithelial cells leading to CD105 expression. To allow AniA activity, the samples were supplemented with 2-mM nitrite, a nitrite concentration that does not compromise the growth of gonococci under aerobic or anaerobic conditions (Figure S2).

During gonococcal infection of the female genital tract, we have shown previously that upregulation of CD105 on vaginal epithelial cells is the decisive host factor, which suppresses epithelial exfoliation (Muenzner et al., 2010). Accordingly, while Opa_{CEA}-expressing, CEACAM-binding bacteria could suppress exfoliation of epithelial cells in CEA-transgenic mice, non-opaque, piliated gonococci induced massive exfoliation as observed by scanning electron microscopy (SEM) of whole mount organs (Figures 5C and 5D). In agreement with the massive exfoliation, only low amounts of piliated, non-opaque bacteria were re-isolated from the genital tract, while higher numbers of Opa_{CEA}-expressing, CEACAM-binding bacteria were recovered (Figure 5E). Interestingly, when recombinant wild-type AniA, but not AniA H280L, was co-applied together with piliated gonococci and 2-mM nitrite, the exfoliation was completely suppressed (Figures 5C and 5D). The reduced detachment of the epithelial cells in the presence of AniA directly correlated with higher numbers of recovered piliated, non-opaque bacteria 24 h after infection (Figure 5E). The lack of exfoliation upon addition of AniA suggests that exogenous production of NO can help to suppress epithelial exfoliation and thereby facilitate bacterial colonization of the vaginal mucosa.

Stimulation of sGC Signaling Suppresses Epithelial Exfoliation *In Vivo*

The striking effect of AniA addition on infection-triggered exfoliation implies that the NO – sGC – PKG – CREB – CD105 axis also plays a role in the intact tissue *in vivo*. Therefore, we reasoned that stimulation of this signaling cascade should suppress the physiological exfoliation response upon infection with non-CEACAM-binding gonococci. Again, female mice were vaginally infected with piliated, non-opaque gonococci, and a strong exfoliation of superficial epithelial cells was observed (Figure 6A). The number of exfoliating cells/area was more than tripled in infected animals compared with the uninfected situation (Figure 6B). In contrast, when the sGC agonist BAY

analyzed by western blotting using antibodies against phosphoCREB (upper panel), total CREB (middle panel), and tubulin (lower panel). The same samples were analyzed by flow cytometry for CD105 expression (right panel).

(B) Cells as in (A) were infected with Ngo Opa_{CEA}, which were treated or not with 4% paraformaldehyde (PFA) prior to infection. Cells were analyzed by flow cytometry for CD105 expression.

(C) Flow chart depicting the denitrification pathway from nitrate to nitrogen. *N. gonorrhoeae* lacks several enzymes and therefore can only utilize nitrite (NO_2-), which is reduced by the nitrite reductase AniA under anaerobic conditions to NO, which can be further converted by the NO reductase NorB to nitrous oxide (N_2O). Other bacteria, such as *E.coli*, can initiate denitrification by using nitrate (NO_3-).

(D) Ngo Opa_{CEA} were cultured under anaerobic growth conditions in the presence of 2 mM $NaNO_2$. Directly after inoculation (T0) and at the indicated days (T2–T5) lysates of the bacteria were prepared and analyzed by western blotting with polyclonal antibodies against AniA (upper panel) or with monoclonal antibodies against Opa proteins (lower panel).

(E) 293T CEACAM1 cells were infected with Ngo Opa_{CEA} and lysates of the bacteria prior to infection (pre) or at the indicated times after infection (post) were analyzed by western blotting as in (D).

(F) Cells as in (E) were infected for 16 h with Ngo Opa_{CEA} grown for the indicated times under anaerobic conditions as in (D). Control cells remained without infection (w/o). Whole cell lysates were analyzed by western blotting using antibodies against phosphoCREB (upper panel), total CREB (middle panel), and tubulin (lower panel).

(G) 293T CEACAM1 cells were infected with Ngo Opa− or Ngo Opa_{CEA} grown for 3 days under anaerobic or for 1 day under regular aerobic conditions as indicated. After 16 h, infected cells were analyzed by flow cytometry for CD105 expression. Each panel shows representative results of at least three independent repetitions.

See also Figure S6.

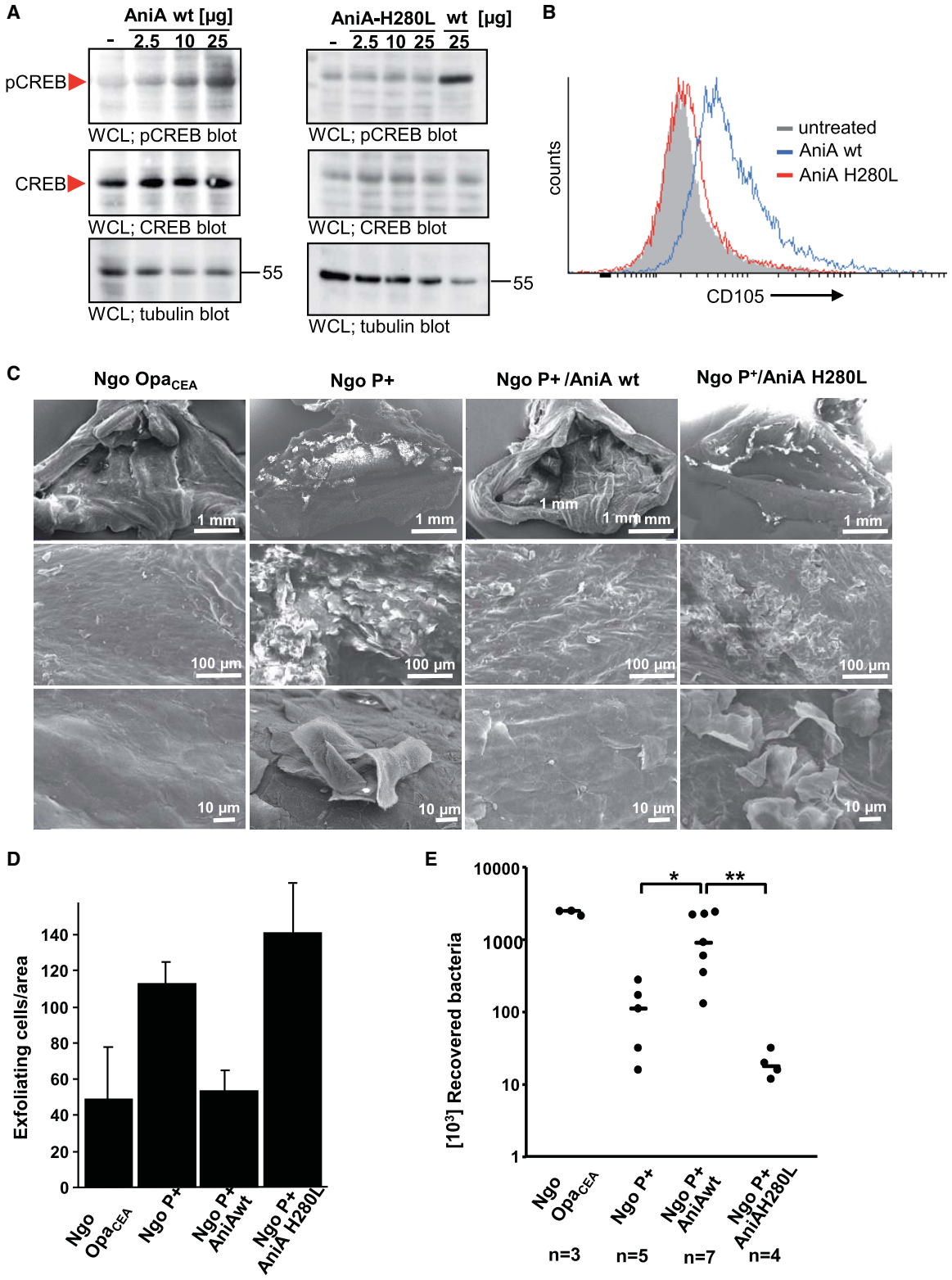

Figure 5. Purified Gonococcal AniA Can Induce CD105 Expression and Suppresses Epithelial Exfoliation

(A) 293T CEACAM1 cells were incubated for 16 h with the indicated amounts of recombinant AniA wild type (WT; left panels) or catalytically inactive AniA H262A (right panels) in the presence of nitrite. Whole cell lysates were analyzed by western blotting using antibodies against phosphoCREB (upper panel), total CREB (middle panel), and tubulin (lower panel). In the right panel, lysates of cells incubated in the presence of 25 µg AniA, WT were loaded as positive control.

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41–2272 was co-applied together with the bacteria, the exfoliation response was blunted and the number of exfoliating cells/area dropped to the level found in uninfected cells (Figures 6A and 6B). As expected, the reduced epithelial exfoliation led to increased numbers of bacteria, which could be recovered from the BAY 41–2272 treated animals (Figure 6C). In mice receiving BAY 41–2272, piliated bacteria could even be observed by immunohistological staining in contact with the vaginal mucosa, while only in very rare occasions piliated, non-opaque gonococci were detected on the mucosa of untreated animals (Figure 6D). Most importantly, CD105 expression was found throughout the mucosal epithelium, when uninfected or infected mice were treated with the sGC agonist (Figure 6D). In contrast, no CD105 expression was observed when mice were infected with non-CEACAM-binding gonococci even in the rare situations, where single bacteria were associated with the mucosa (Figure 6D). These results corroborate the prior *in vitro* findings and link the sGC – cGMP-PKG signaling axis to CD105 expression by vaginal epithelial cells *in vivo*.

Blockade of Host Cell sGC Signaling Interferes with Gonococcal Colonization by Re-establishing Epithelial Exfoliation

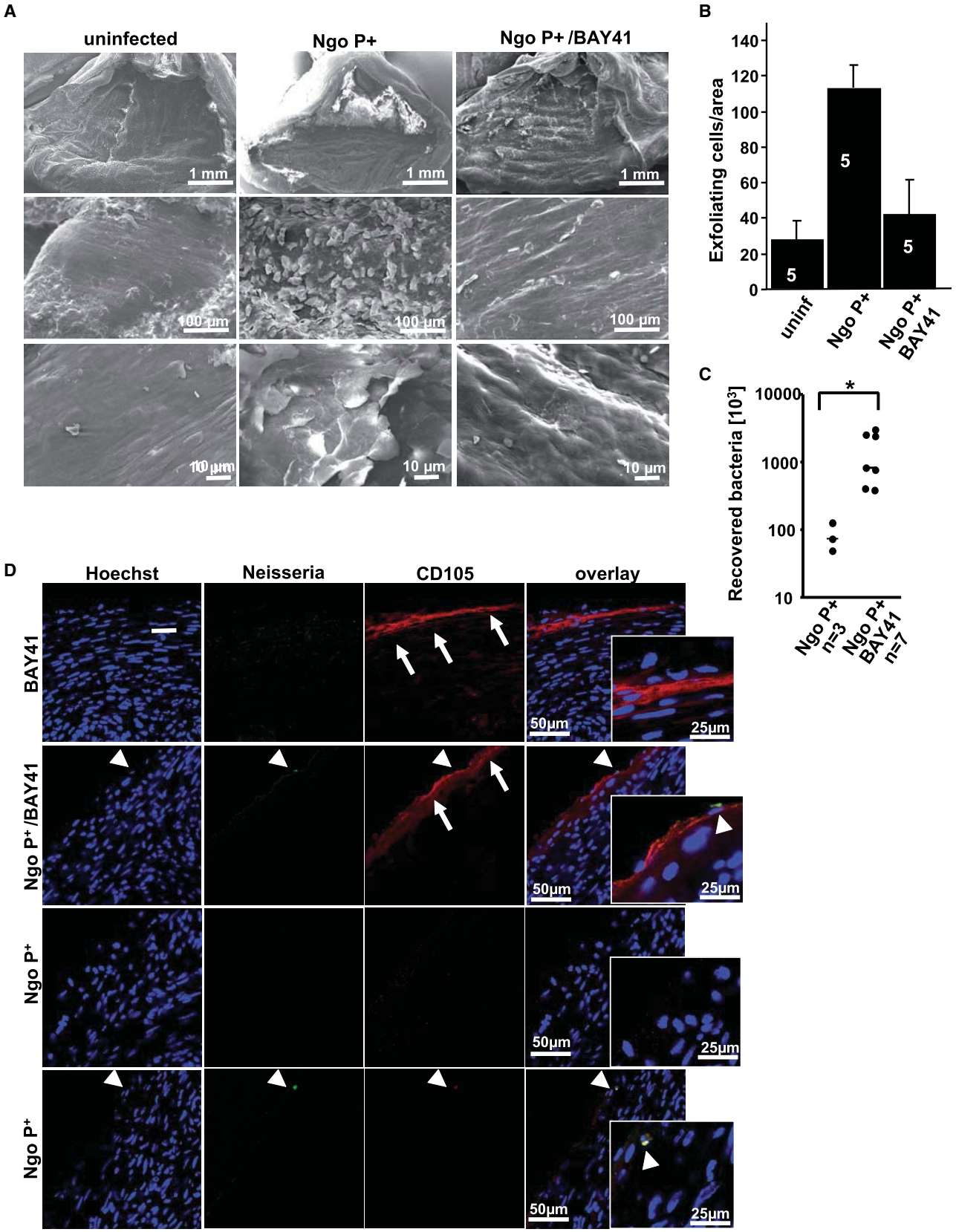
As gonococci appear to profit from the suppression of exfoliation, it would be desirable to stimulate epithelial exfoliation as a means to reduce cell-associated bacteria and to counteract mucosal colonization by these highly adapted pathogens. Therefore, we asked whether an antagonist of sGC could revert the bacteria-induced suppression of exfoliation. Accordingly, we applied the sGC inhibitor ODQ during vaginal infection. As reported before and in contrast to the non-CEACAM-binding gonococci (see Figure 6A), the Opa_{CEA}-expressing, CEACAM-binding gonococci almost completely suppressed exfoliation, with numbers of exfoliating cells comparable to uninfected animals (Figures 7A and 7B). However, upon co-application of ODQ, a strong exfoliation response of the superficial epithelial cells was observed (Figure 7A). The increased exfoliation seen in ODQ-treated animals was not elicited by the inhibitor, as uninfected animals treated with ODQ did not show signs of increased exfoliation (Figure 7A). The numbers of exfoliating cells/area in the animals infected for 24 h with Opa_{CEA}-expressing bacteria and treated with ODQ were 3-fold higher than in uninfected animals or in animals treated with solvent only (Figure 7B). In the solvent-treated animals, CEACAM-binding bacteria could be found in association with the mucosal surface on almost every immunohistological tissue section, while in the ODQ-treated animals only occasionally bacteria could be detected (Figure 7C). As expected, the untreated animals expressed CD105 on their superficial epithelial cells 24 h after infection with CEACAM-binding bacteria (Figure 7C). In contrast, in the ODQ-treated animals not only lower numbers of bacteria remained on the epithelial surface but also CD105 expression by the host cells was not observed (Figure 7C). Most likely as a consequence of the increased exfoliation upon ODQ application, lower numbers of bacteria could be re-isolated 24 h after infection, despite the fact that Opa_{CEA}-expressing bacteria were able to attach to the human host receptor present in the CEA-transgenic mice (Figures 7C and 7D). Together, these results underscore the role of the bacteria-triggered, NO-mediated host cell signaling cascade for CD105 expression by superficial epithelial cells and the suppression of exfoliation. Furthermore, the striking reversion of the tissue phenotype and the reduced bacterial recovery upon ODQ treatment suggests that local pharmacological intervention into a host signaling pathway might be an effective means to interfere with gonococcal colonization of the female genital tract.

DISCUSSION

Neisseria gonorrhoeae is a highly specialized microbe, which does not occur outside of the human body. Therefore, survival of gonococci strictly depends on the ability of these bacteria to colonize the mucosal surface of their sole natural host. The low dose of less than 1,000 gonococci needed for a productive infection in men (Schneider et al., 1995) attests to the remarkable efficiency by which *N. gonorrhoeae* can establish itself on mucosal surfaces of the urogenital tract, the throat, the colon, or the eye. Gonococci, similar to other human-restricted microbial pathogens, engage epithelial receptors of the CEACAM family to intimately attach to the mucosal surface of the vaginal tract (Islam et al., 2018; Sintsova et al., 2015). Aside from physical attachment, CEACAM binding also facilitates colonization by allowing the bacteria to suppress epithelial exfoliation (Muenzner et al., 2010, 2005). Here, we identify an unexpected communication between CEACAM-associated *N. gonorrhoeae* and the mucosal epithelium, which helps to explain the extraordinary capability of gonococci to colonize the human mucosa. Our results indicate that an intimate Opa adhesin-mediated interaction with CEACAM family receptors is pre-requisite to allow a short-range messenger and metabolic intermediate of bacterial anaerobic respiration, NO, to reach the host cell. Inside the eukaryotic cell, NO triggers a conserved signaling cascade involving sGC, cGMP, PKG, and the transcription factor CREB to upregulate expression of CD105, which is responsible for increased cell-matrix adhesion and blunted epithelial exfoliation.

Host-produced NO is known to have antibacterial properties in inflammatory settings. Indeed, the iNOS, which can be

(B) Cells were incubated as in (A) with 25 μg of AniA WT, AniA H262A, or left untreated. Then, cells were analyzed by flow cytometry for CD105 expression.
(C) CEA-transgenic (CEAtg) mice were infected with Opa_{CEA}-expressing gonococci (Ngo Opa_{CEA}), non-opaque, piliated gonococci (Ngo P+), or infected with Ngo P+ supplemented with 25 μg active (WT) or inactive (H262A) AniA in the presence of nitrite. 24 h after infection, the urogenital tracts were excised and processed for SEM. SEM pictures at different magnifications show whole mount organs (top row) or the luminal surface of the upper vaginal and cervical regions.
(D) In samples from (C), the number of exfoliating cells/area was enumerated. Bars represent mean ± SD of exfoliating cells from an area of ~0.075 mm^2 (n = 24; 8 areas/animal) derived from three animals each treatment.
(E) CEAtg mice were infected as in (C) and 24 h later, bacteria were re-isolated from the genital tract. Each data point in the graph reflects the number of bacteria re-isolated from an individual animal (n = 3–7). Data were compiled from two independent experiments. The median for each experimental group of animals is indicated by a line; numbers of recovered bacteria were compared by Mann–Whitney U test and significant differences (**$p < 0.01$, *$p < 0.05$) are indicated.
See also Figures S2 and S7.



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expressed by stimulated macrophages, rapidly produces micromolar amounts of NO radicals by conversion of arginine to citrulline (Aktan, 2004). While high concentrations of the NO radical can be detrimental to microorganisms and eukaryotic cells alike (Fang, 2004), the nanomolar concentrations of NO produced by constitutive NOS (cNOS) enzymes, which are expressed by several cell types, have important physiological roles. In particular, cNOS derived NO stimulates the activity of sGC, which produces the intracellular second messenger cGMP to regulate a multitude of homeostatic processes in mammals such as blood pressure, neurotransmitter release, or platelet aggregation (Alderton et al., 2001; Ignarro, 1990). Both mammalian cNOS and iNOS alike are sensitive to inhibition by the arginine antagonist ADMA. It was therefore surprising to observe that the bacteria-induced upregulation of CD105 depends on a NO-triggered signaling cascade, yet this process is undisturbed by ADMA. These puzzling findings can now be reconciled by the fact that the NO production, which leads to CD105 expression, does not rely on a host-derived NOS enzyme, but rather is due to the gonococcal copper-containing nitrite reductase AniA (Boulanger and Murphy, 2002; Mellies et al., 1997). This enzyme is expressed by gonococci, when oxygen as an electron acceptor is limited and the bacteria instead utilize nitrite in a truncated denitrification pathway (Barth et al., 2009). Identifying the anaerobic metabolism of gonococci as the source of NO not only nicely explains the lack of CD105 expression in response to physiological CEACAM stimulation but also helps to clarify two further enigmatic features of bacteria-triggered CD105 upregulation and suppression of exfoliation: the requirement for intimate, Opa-CEACAM-mediated attachment of the pathogens as well as the potentiation of the phenotype by anaerobic pre-conditioning of the bacteria. In this context it is important to stress that NO is able to freely traverse biological membranes but can only act on short distances (Furchgott and Jothianandan, 1991). The limited working range of NO within a few micrometers is due to the radical nature of this gas, as it rapidly reacts with thiol groups, iron-sulfur clusters in proteins, and, in aerobic environments, with reactive oxygen species (Grisham et al., 1999). Therefore, the intimate interaction afforded by the Opa-CEACAM interaction, which leads to a tight embedding of the bacteria into the host cell membrane or even to endocytic uptake into the host cell (Schmitter et al., 2007; Wang et al., 1998), appears as a pre-requisite to allow sufficient amounts of NO to reach the host cell cytoplasm and to initiate NO-sensitive signaling pathways. Accordingly, non-Opa-expressing bacteria, which do not tightly associate with the host cell surface, are not in a position to influence host processes via NO. A critical parameter influencing the ability of gonococci to suppress exfoliation is the absence of oxygen and the switch of these bacteria to anaerobic respiration. In this regard, AniA is one of the major proteins expressed under low oxygen conditions as found in the female genital tract, a habitat rich in facultative and obligate anaerobic organisms (Anahtar et al., 2015; Ravel et al., 2011). As gonococci are generally cultured under microaerophilic conditions, AniA expression is low to absent under such conditions. However, upon oxygen depletion, AniA expression is massively upregulated and the presence of AniA-directed antibodies in the sera of gonorrhoea patients suggests that this process is relevant *in vivo* (Clark et al., 1988). Moreover, we find AniA expressed within a few hours after infection of isolated epithelial cells, suggesting that static cell cultures attain anaerobic conditions during infection assays *in vitro*. These findings also clarify, why bacteria-triggered upregulation of CD105 by isolated epithelial cells occurs within several hours and not within minutes of infection (Muenzner et al., 2005), as bacteria first have to get exposed to decreasing oxygen levels in the infected cultures. However, pre-conditioning of the bacteria by anaerobic growth conditions or addition of exogenous, recombinant AniA can directly trigger NO-signaling in the eukaryotic cells and exaggerate CD105 upregulation.

Though bacterially derived metabolites such as short chain fatty acids (SCFAs) are known to influence host cells (Donohoe et al., 2011; Fellows et al., 2018; Kelly et al., 2015), the direct signaling activities of a bacteria-derived gas have not been observed before. In contrast to stable molecules such as SCFAs, which can be consumed by co-occurring commensal bacteria or can act on distant tissues, the short-range action of the NO radical assures that only the cell-attached NO-producing gonococci will directly profit from the exchange with the epithelium. That the resulting suppression of exfoliation might be particularly useful in colonizing the urogenital tract is demonstrated by a natural experiment: recent years have seen the emergence of a novel group of *Neisseria meningitidis*, which represents the closest relative of gonocci and usually inhabits the nasopharynx of humans, where it is transmitted via aerosols. This arising clonal complex of meningococci has acquired the ability to colonize the urogenital tract and to be transmitted by sexual contact, generating a new venereal disease (Bazan et al., 2016). The

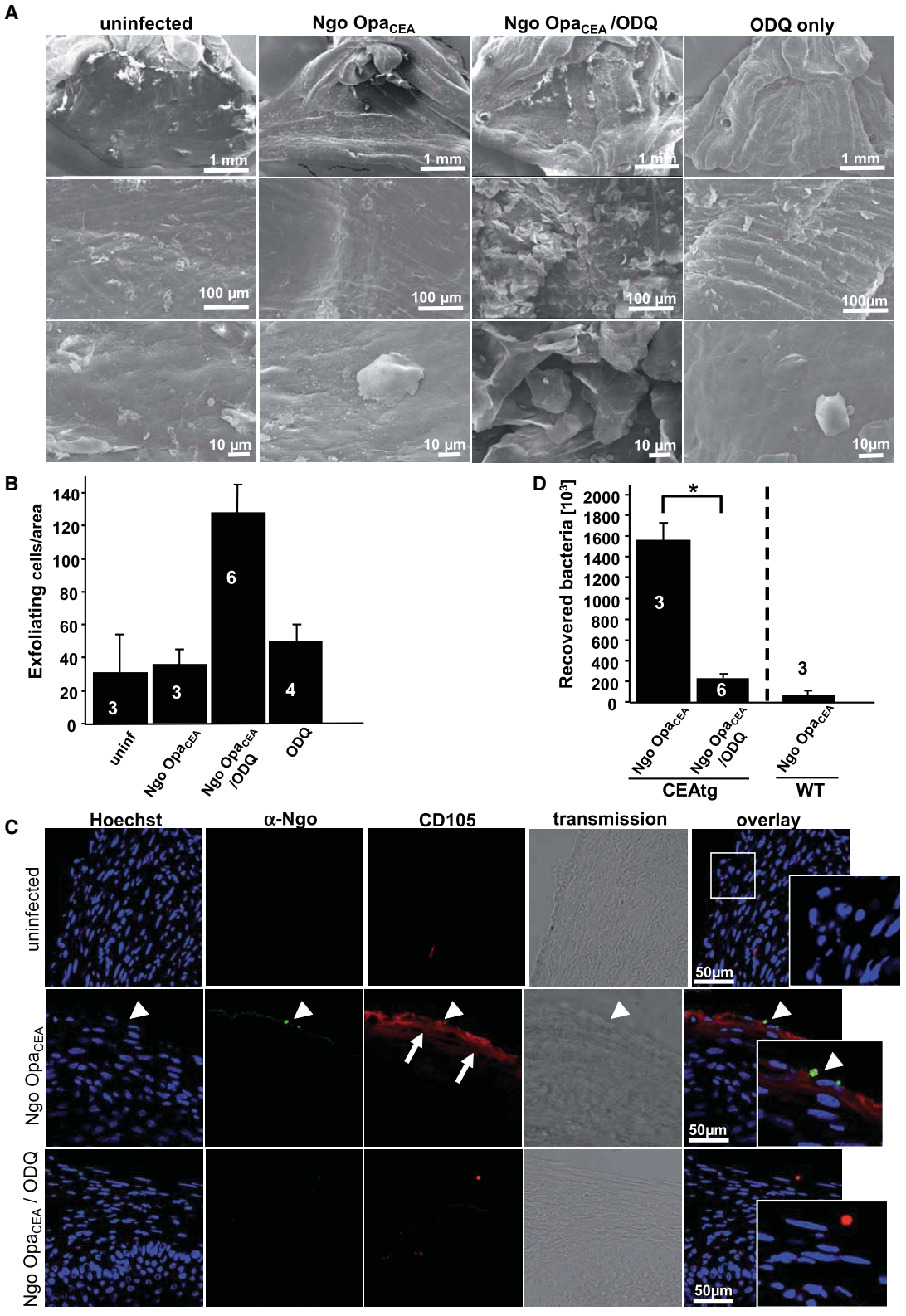
Figure 6. Stimulation of sGC Signaling Suppresses Epithelial Exfoliation *In Vivo*

(A) CEAtg mice were treated or not with 50 nM BAY41-2272 (BAY41) 30 min before infection with piliated, non-opaque (Ngo P+) gonococci. Some untreated animals were left uninfected. After 24 h, the urogenital tracts were excised and processed for SEM. SEM pictures at different magnifications show whole mount organs (top row) or the luminal surface of the upper vaginal and cervical regions. Whereas massive epithelial exfoliation is evident for CEAtg mice infected with pilliated gonococci, a strongly reduced detachment of epithelial cells is observed in mice pretreated with the sGC stimulator.

(B) Quantification of exfoliating epithelial cells/area from samples in (A). Bars represent mean ± SD of exfoliating cells from an area of ~0.075 mm^2 with 5 separate areas per organ and 5 animals per treatment (n = 25).

(C) CEAtg female mice were infected as in (A) and 24 h later, bacteria were re-isolated from the genital tract. Each data point in the graph reflects the number of bacteria re-isolated from an individual animal (n = 3–7). Data were compiled from two independent experiments. The median for each experimental group of animals is indicated by a line; numbers of recovered bacteria were compared by Mann–Whitney U test and significant differences (*$p < 0.05$) are indicated.

(D) Genital tracts from CEAtg mice infected for 24 h with Ngo P+ in the presence or absence of BAY41 or from uninfected animals treated with BAY41 were excised and cryosections were co-stained with rabbit polyclonal antiserum against *N. gonorrhoeae* (green) and against murine CD105 (red). Cell nuclei were visualized by Hoechst (blue). Arrowheads indicate bacteria in contact with the epithelial surface. Host-associated bacteria rarely occurred in untreated animals, where most tissue sections did not contain bacteria (see the two bottom rows with sections from untreated animals). Expression of CD105 (white arrows) is seen in mice treated with BAY41 irrespective of infection, while CD105 is absent in untreated animals. The inserts show magnifications of bacterial attachment sites.



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astonishing change in lifestyle of these meningococci is accompanied by gene losses, but also by the acquisition of a large, 3.8-kb gonococcal genomic fragment encoding the AniA and NorB genes (Tzeng et al., 2017). Our results now provide a causal explanation, how AniA and its NO-producing activity can facilitate mucosal colonization in the urogenital tract by both gonococci and menigococci.

The central role that AniA has in this process renders this enzyme an interesting target for vaccine development or even direct pharmacological inhibition. A more immediate access point for interference with this process is provided by the well-developed suite of pharmacological agents that modulate the human NO-signaling cascade, such as agonists and antagonists of the sGC. Indeed, topical application of an sGC inhibitor to the female genital tract disrupts the ability of the bacteria to suppress epithelial exfoliation. In the case of *Neisseria gonorrhoeae*, where antibiotic treatment options are getting limited and where the transmission event usually does not occur unnoticed, such a kind of post-exposure treatment could block the initial stages of the infection process. Therefore, our study not only provides a surprising additional chapter to the means by which prokaryotes communicate with their multicellular hosts but also provides an unexpected opportunity to disrupt the chain of transmission for one of the major venereal diseases worldwide.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

Conceptualization, P.M. and C.R.H.; Methodology, P.M. and C.R.H.; Investigation, P.M.; Writing, C.R.H.; Funding Acquisition, C.R.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure 7. Blockade of Host Cell sGC Signaling Interferes with Gonococcal Colonization by Re-establishing Epithelial Exfoliation

(A) CEAtg mice were infected for 24 h with Opa_{CEA}-expressing gonococci (Ngo Opa_{CEA}) in the presence or absence of ODQ (50 nM) or left uninfected. As indicated some uninfected mice were also treated with ODQ. 24 h after treatment, the urogenital tracts were excised and processed for SEM. SEM pictures at different magnifications show whole mount organs (top row) or the luminal surface of the upper vaginal and cervical regions.

(B) Quantification of exfoliating epithelial cells from samples in (A). Bars represent mean ± SD of exfoliating cells from an area of ~0.075 mm^2 with 5 separate areas per organ and 3–6 animals each treatment as indicated.

(C) CEAtg mice were infected for 24 h with Ngo Opa_{CEA} gonococci in the presence or absence of ODQ. Cryosections of the urogenital tract epithelium were co-stained with antibodies against *N. gonorrhoeae* (green) and against murine CD105 (red). Cell nuclei were visualized by hoechst (blue). Arrowhead indicates bacteria in contact with the epithelial surface. Expression of CD105 is seen in infected mice (white arrows) but not upon treatment with ODQ.

(D) CEAtg female mice were infected with Ngo Opa_{CEA} in the presence or absence of ODQ. As a further control, wild-type mice were infected with Ngo Opa_{CEA}. 24 h later, bacteria were re-isolated. Bars represent the mean number ± SD of bacteria re-isolated from individual animals (n = 3 or 6). Data were compiled from two independent experiments. The numbers of bacteria recovered from CEAtg mice were compared by Kruskal-Wallis one-way analysis of variance on Ranks and significant differences are indicated (*$p < 0.05$).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-tubulin (clone E7)	DSHB, University Iowa	AB_528499
Mouse monoclonal anti-CEACAM (clone D14HD11)	Aldevron	Cat # GM-0505
Mouse monoclonal anti-CEACAM (COL-1)	Zymed	No. 20973473
Rabbit polyclonal anti-phospho-CREB (Ser 133)	Cell Signaling Technology	Cat # 8763
Mouse monoclonal anti-CREB (clone 86B10)	Cell Signaling Technology	Cat # 9104
Mouse monoclonal anti-CD105/Endoglin (clone P4A4)	DSHB, University of Iowa	AB_528222
Rat Anti-Mouse anti-CD105/Endoglin (clone MJ7/18)	SouthernBiotech	Cat # 1860-01
Mouse monoclonal anti-HA (clone 12CA5)	Purified from hybridoma supernatant	AB_2532070
Rabbit polyclonal anti-AniA	this manuscript	N/A
Rabbit polyclonal anti-FAK (A-17)	Santa Cruz Biotechnology	sc-557
Rabbit polyclonal anti-*N.gonorrhoeae*	Immunoglobe, Himmelstadt, Germany	IG-511
Mouse monoclonal anti-Opa; clone 4B12/C11	DSHB, University of Iowa	opa 4B12-
Mouse monoclonal anti-GFP clone JL-8	BD Biosciences	AB_2314359
DyLight 405-conjugated goat anti-mouse	Jackson ImmunoResearch	AB_2338791
Cy2-conjugated goat anti-rabbit	Jackson ImmunoResearch	AB_2338019
Cy3-conjugated goat anti-mouse	Jackson ImmunoResearch	AB_2338685
HRP-conjugated goat anti-mouse	Jackson ImmunoResearch	AB_2307392
HRP-conjugated goat anti-rabbit	Tebu-bio, Offenbach, Germany	L315
Bacterial Strains		
Non-piliated *N.gonorrhoeae* MS11-B2.1 Opa52	T.F. Meyer, MPI for Infection Biology, Berlin, Germany	N309
Non-piliated *N.gonorrhoeae* MS11-B2.1 Opa$^-$	T.F. Meyer, MPI for Infection Biology, Berlin, Germany	N302
Piliated *N.gonorrhoeae* MS11 Opa-/P+	T.F. Meyer, MPI for Infection Biology, Berlin, Germany	N280
Escherichia coli AfaE-III	Muenzner et al., 2016	A30
Escherichia coli ΔAfaE-III	Muenzner et al., 2016	A30-1
Escherichia coli BL21(DE3)	Novagen, Madison, Wisconsin	Cat # 69450
Inhibitors		
PKG inhibitor; 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphothioate Rp-isomer	Calbiochem, Darmstadt, Germany	Cat # 370655
PKA inhibitor; 14-22 Amide	Calbiochem	Cat # 476485
PKC inhibitor; chelerythrine chloride	Calbiochem	Cat # 220285
CaMKII iInhibitor; KN-62	Calbiochem	Cat # 422706
sGC inhibitor; oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ)	Calbiochem	Cat # 495320
sGC agonist; BAY 41-2272	Calbiochem	Cat # 196876
iNOS inhibitor; N,N-dimethyl-L-arginine (ADMA)	Cayman Chemical (BIOMOL GmbH, Hamburg, Germany	Item No. 80230
DETA Nonoate	Cayman Chemical	Item No. 82120
Recombinant Proteins, Chemicals		
recombinant AniA wt; AniA H280L	this manuscript	N/A
17-β-Estradiol	Calbiochem	Cat # 3301
Trimethoprim 98%	Acros Organics, Geel, Belgium	Cat # 455120050
IPTG	Roth	Cat # 206-703-0

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
GC-Agar	Becton Dickinson	Cat # 228950
OptiMEM	Life Technologies, Darmstadt, Germany	Cat # 31985062
DMEM	Merck	Cat # FG0435
Fetal calf serum	Biochrom	Cat # S0115
D-luciferin	Thermo Fisher Scientific	Cat # L2916
Griess reagent kit	LifeTechnologies	Ca t# G7921
Tissue-Tec	Cryo-M-Bed; Bright Instrument, Huntingdon, UK	No. 1321700001
Plasmids		
Constitutive active PKG Ia	Deguchi et al., 2002	Addgene (Cambridge, MA) # 16392
Constitutive active PKG Ib	Deguchi et al., 2002	Addgene (Cambridge, MA) # 16396
pRSV CREB S133A (dominant-negative CREB)	Gonzalez and Montminy, 1989	Addgene (Cambridge, MA) # 22395
psPAX2	psPAX2 was a gift from Didier Trono	Addgene (Cambridge, MA) # 12260
pMD2.G	pMD2.G was a gift from Didier Trono	Addgene (Cambridge, MA) # 12259
pLL3.7	Rubinson et al., 2003	Addgene (Cambridge, MA) # 11795
pGL-2-basic Luciferase, promoterless	Promega	E1641
pDNR-dual-LIC	Adrian et al., 2019	N/A
pET24a-His-SUMO-loxP	this manuscript	N/A
pET24a-His-SUMO-loxP Ani wt / AniA H280L	this manuscript	N/A
pEGFP murine FAK (GFP-FAK)	Dusko Ilic, UCSF, CA Agerer et al., 2005	N/A
pcDNA3.1 HA-tagged murine FAK (HA-FAK)	David Schlaepfer, UCSD, CA Agerer et al., 2005	N/A
CD105 promoter constructs pCD105FL, pCD105s	Carmelo Bernabeu, Madrid, Spain Botella et al., 2002	N/A
pCD105FL ΔCRE1	this manuscript	N/A
pcDNA3.1 CEACAM1-4L-HA	Muenzner et al., 2016	N/A
pLL3.7 CEACAM1ΔCT-GFP	this manuscript	N/A
Cell Lines		
HEK293T	DSMZ, Braunschweig, Germany	ACC-635
Human vaginal epithelial cells (hVEC) MS74	A.J. Schaeffer, Chicago, IL Rajan et al., 2000	N/A
ME-180	ATCC, Rockville, MD	ATCC HTB-33
Software and Algorithms		
SkanIT software	ThermoScientific	Version 2.4.3
FACS Diva software	BD Biosciences	Version 8.0.1
Adobe Photoshop	Adobe	Version CS4
Powerpoint Professional Plus	Microsoft	Version 2016
Cyflogic	Cyflogic	Version 1.2.1
FlowJo	Tree Star	Version 10.0.7
SigmaStat for Windows	SysStat	Version 4.0

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to the Lead Contact, Christof R. Hauck (christof.hauck@uni-konstanz.de). Unique plasmids generated in this study are available without restriction. There are restrictions to the availability of newly generated recombinant proteins and polyclonal antibodies as well as cell lines due to the limited amounts available. We are glad to share those reagents with reasonable compensation by requestor for its processing and shipping.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

C57BL/6J mice carrying the complete human CEA gene (CEAtg mice) (Eades-Perner et al., 1994) and wildtype C57BL/6J mice (obtained from Elevage Janvier, Le Genest Saint Isle, France) were kept under specified pathogen-free conditions under a 12-h light cycle in the animal facility of University of Konstanz in accordance with national and institutional guidelines. The CEAtg line was maintained heterozygous for the CEA transgene by crossing male CEAtg mice with female wildtype mice. Offspring of these crosses were genotyped by PCR and female mice were used between 6 – 8 weeks of age for infection experiments. Experiments involving animals were performed in accordance with the German Law for the Protection of Animal Welfare. The animal care and use protocol, including the protocol of experimental vaginal infection of female mice, was approved by the appropriate state ethics committee and state authorities regulating animal experiments (Regierungspräsidium Freiburg, Germany) under the permit file number G-15/43.

Bacterial Culture

Gonococcal strains used in this study were derived from *N. gonorrrhoeae* strain MS11 and were described previously (Muenzner et al., 2005). Strains were routinely cultured at 37°C in 5% CO_2 on GC agar plus vitamin supplement (Becton Dickinson) and passaged daily. The gonococci were either non-piliated and expressed a CEACAM-binding Opa protein (Ngo Opa_{CEA}; strain N309), they were non-piliated and non-opaque (Ngo Opa-; strain N302), or they were piliated and non-opaque (Ngo P+; strain N280).

For infection with aerobic grown cultures, the bacteria were suspended in DMEM, the optical density of the suspension at 550nm (OD_{550}) was used to estimate the number of the microorganisms according to a standard curve, and the bacteria were added to the cells at the indicated multiplicity of infection (MOI) or were used for vaginal infection of mice using 1 x 10^8 bacteria/animal.

Anaerobic cultivation of gonococci was performed in anaerobic chambers containing GasPak™ EZ Anaerobe Container System Sachets (Becton-Dickinson, Heidelberg, Germany) or in liquid culture. In the first case, the gonococci were inoculated onto GC agar, and a sterile filter disk (Schleicher and Schuell) containing 20 μl of a 20% $NaNO_2$ solution placed in the center of each plate (Knapp and Clark, 1984). To create low oxygen liquid cultures, 20 ml of PPM medium was supplemented with 2 mM $NaNO_2$ and filled into 25 ml McCartney bottles. Bacteria were added to an OD_{550} of 0.1, bottles were closed, and incubated on a shaking platform (90 rpm) at 37° C for different time points. To analyse the gonococci for the presence of Opa protein, single colonies were expanded on GC agar, lysed and analysed by Western blotting using Opa specific antibodies (clone 4B12/C11).

The uropathogenic *E. coli* strain A30 (isolated from a cystitis patient) harbouring the *afa-3* gene cluster is a non-hemolytic, serotype O75 strain expressing the CEACAM-binding AfaE-III adhesin (*E. coli* AfaE-III). *E. coli* AfaE-III and the *afa*-deficient isogenic strain (*E. coli* ΔAfaE-III) have been described previously (Muenzner et al., 2016) and were cultured on LB agar.

Cell Culture

The human embryonic kidney cell line 293T (293T cells; ACC-635, DSMZ, Braunschweig, Germany) was cultured and transiently transfected as described (Muenzner et al., 2005).

The human cervix carcinoma cell line ME-180 (ATCC HTB-33, Rockville, MD) was cultured in DMEM containing 10% fetal calf serum (FCS) at 37°C in 5% CO_2 and subcultured every third day. The human vaginal epithelial cell (hVEC) line MS74 (Rajan et al., 2000) was obtained from A.J. Schaeffer (Feinberg School of Medicine, Northwestern University, Chicago, IL), cultured on gelatine-coated dishes in DMEM containing 10% FCS, non-essential amino acids, pyruvate and subcultured every third day.

METHOD DETAILS

Generation of Stable Cell Lines

To generate a CEACAM1ΔCT-GFP encoding lentiviral vector, human CEACAM1 cDNA was amplified from plasmid pcDNA3.1 CEACAM1-4L-HA (Muenzner et al., 2016) with the forward primer CEACAM1-Nhe_sense 5'-GAACTGCTAGCACCATGGGGCACCTCTCAG-3' and reverse primer CEACAM1ΔCT-AgeI_reverse 5'-GCTAGACCGGTGCATATTTCCCGAAATGCAG-3'. The resulting fragment was cloned in the NheI and AgeI restriction sites of lentiviral vector pLL3.7 (Rubinson et al., 2003) resulting in an in-frame fusion between CEACAM1 extracellular and transmembrane domains and the GFP coding sequence. To generate lentiviral particles, 293T cells were transfected using calcium phosphate DNA precipitation with 5 μg of pLL3.7 CEACAM1ΔCT-GFP, 3 μg of packaging plasmid psPAX2 and 2 μg of plasmid pMD2.G encoding the VSV envelope protein (Addgene plasmids #12260 and #12259). After 8 h, the medium was replaced with fresh DMEM containing 10 %CS. Three days later, the virus-containing culture medium was collected, centrifuged at 2000 rpm at 4°C for 7 min, sterile filtered and stored. HEK 293 were transduced with lentiviral particles and 3 days later sorted by FACS to select for GFP-positive cells. The sorted, GFP-positive population of CEACAM1ΔCT-GFP expressing 293T cells (CEACAM1 cells) was used for experiments.

Immunofluorescence Staining, Western Blotting and Flow Cytometry

Western blotting and immunofluorescence staining was performed as described previously (Schmitter et al., 2004). Flow cytometry was performed on a LSRII flow cytometer (BD Biosciences) equipped with 405nm, 488nm, and 561nm laser lines using FACS Diva software.

Production of Soluble CEACAM N-Terminal Domains and Binding to Bacteria

293T cells were transfected with 5 μg plasmid DNA encoding secreted GFP-fusion proteins of the N-terminal domains of human CEACAM family members. After 24 h, the medium of the transfected cultures was replaced by OptiMEM (LifeTechnologies, Darmstadt, Germany). Two days later the culture supernatants containing the secreted fusion proteins were collected, centrifuged for 15 min at 5000 rpm. The GFP-derived fluorescence was analysed using a Varioskan Flash reader (ThermoScientific) to adjust equal amounts of the secreted fusion proteins. For pull-down experiments, indicated bacteria were suspended in PBS and binding to the receptor protein contained in cell culture supernatants was determined as described (Kuespert et al., 2007).

Cell Adhesion Assay

To evaluate cell adhesion to the extracellular matrix, 96-well plates were coated with 25 μg/ml collagen type 1 from calf skin (ICN Biomedicals, Irvine, CA) or BSA in PBS for 24 hours at 4°C. 293T CEACAM1 cells were serum starved (20 h) and infected with the indicated bacterial strains at a MOI of 30 for 16 hours. Following infection, cells were detached and kept in suspension medium (DMEM, 0.2% BSA) for 15 min before being replated at 4 x 10^4 cells/well onto the collagen-coated or BSA-coated wells. Cells were allowed to adhere for 90 min before non-adherent cells were removed by three gentle washes with PBS. Remaining cells were fixed and stained for 60 min with 0.1% crystal violet in 0.1 M borate, pH 9. After washing and drying, the crystal violet was eluted in 10 mM acetic acid and the staining intensity was measured at 590 nm with a Varioskan Flash (ThermoScientific, Oy Microplate Instrumentation; Vantaa, Finland).

CD105 Promoter Constructs and Luciferase Assays

The CD105 promoter constructs pCD105FL and pCD105s correspond to pCD105(-1950/+350) and pCD105(-50/+350), respectively (Botella et al., 2002), and were generously provided by Carmelo Bernabeu (Centro de Investigaciones Biológicas, CSIC, Madrid, Spain). Mutation of the CRE consensus binding site 1 (Graulich et al., 1999) was achieved with primers ΔCRE1-for 5'-GGCAGACG GATCACTTGAATTCAGGAGTTCGAGACCAGC-3' and ΔCRE1-rev 5'- GCTGGTCTCGAACTCCTGAATTCAAGTGATCCGTCTGCC -3'. The mutation was confirmed by DNA sequence analysis. For luciferase assays, 293T CEACAM1 cells were co-transfected with 4 μg of different CD105 reporter constructs together with 1 μg mKate-encoding plasmid for normalization. As a negative control, pGL-2 basic encoding a promoterless luciferase was used (Promega, Cambridge, USA). In some cases, cells received additionally plasmids encoding caPKGIα or CREB S133A. Then the remaining samples were additionally transfected with the empty vector control (pcDNA) to end up with the same total quantities of plasmid DNA. 24 h after transfection, cells were either infected or not for 16 hours with gonococci cultured under anaerobic growth conditions, or cell were treated or not with the sGC stimulator BAY, the NO releasing substance DETA (for 6 hours). After washing, cells were lysed with 300 μl of lysis buffer (25 mM Tris/phosphate, 4 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM dithiothreitol) for 15 minutes at 4°C. After centrifugation at 13,000 rmp for 5 minutes, 30 μl of each supernatant was transferred to a 96-well plate and 175 μl assay buffer (25 mM Tris/phosphate, 20 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM dithiothreitol) and 1 mM D-luciferin were added and measured over 10000 ms, in a Varioskan® Flash (ThermoFisher) using SkanIT software.

Cloning and Expression of Recombinant AniA

The *aniA* gene of *N. gonorrhoeae* MS11-B2 was amplified from genomic DNA using primers AniA_for 5'-ACTCCTCC CCCGCCATGGGTGGCGAACAAGCTGC-3' and AniA_rec 5'-CCCCACTAACCCGTTAATAAACGCTTTTTTCGGATGCAGAGGC-3'. The PCR product was cloned into a pDNR-dual-LIC vector (Adrian et al., 2019) to yield plasmid pDNR-dual-LIC-AniA. Using the primers AniA H280L_for 5'-GGCAGACGGATCACTTGAATTCAGGAGTTCGAGACCAGC-3' and AniA H280L_rev 5'-GCTGGTCTCGAACTCCTGAATTCAAGTGATCCGTCTGCC-3' a missense mutation was introduced into AniA WT to yield the catalytically inactive AniA H280L. Via Cre mediated recombination, the cDNAs were transferred into the acceptor vector pET24a-His-SUMO-loxP, which was generated from vector pCA528 (Andréasson et al., 2008) by insertion of a LoxP cassette in-frame with the His_6-SUMO coding sequence (Creator-acceptor-vector-construction Kit; BD Biosciences). AniA wt and AniA H280L were expressed in *E. coli* BL21(DE3) at 30°C for 4h upon induction with IPTG. The proteins were purified from bacterial lysates by standard Ni-affinity chromoatography and the size and purity of AniA was analysed by SDS PAGE.

AniA Activity Assay

Nitrite reduction by AniA was evaluated using the Griess reagent kit (LifeTechnologies) according to (Kahler and Guze, 1957). Briefly, recombinant AniA wildtype or AniA H280L (20 μg) were incubated at 30°C in 20 mM phosphate buffer pH 6.0 with 2 mM $NaNO_2$, 14 mM sodium dithionite, and 0.7 mM methyl viologen under anaerobic conditions. After 5, 15, 30, 60, 120, 180 and 240 min, the reaction was stopped and 50 μl of the samples were transferred to triplicate wells of a 96-well plate. There, 50 μl of 1% sulfanilamide in 5% phosphoric acid were added and after 5 min incubation mixed with 50 μl of 0.1% N-1-napthylethylenediamine dihydrochloride in H_20. After 10 min, the absorption at 548nm was determined to calculate the remaining nitrite in the sample according to a standard curve obtained with $NaNO_2$.

Vaginal Infection with *N. gonorrhoeae*

Experimental vaginal infection of female mice with gonococcal strains was performed as previously described (Muenzner et al., 2010). Briefly, female mice were subcutaneously injected with 17-β-estradiol (Calbiochem, Darmstadt, Germany) 4 days prior to

infection. Trimethoprim sulphate was administered orally via drinking water (40 mg/ 100 ml) to reduce growth of commensal flora during estrogen treatment. Before infection, the vagina was washed three times with 30 μl PBS and mice were inoculated intravaginally with 10^8 CFU of the indicated bacteria suspended in 20 μl of PBS. Pharmacological compounds (BAY 41-2272 or ODQ) were applied vaginally 10 min prior to the infection in a volume of 20 μl. Recombinant AniA (25 μg) was inoculated together with the bacterial suspension in PBS and supplemented with 2 mM $NaNO_2$. 24 h after infection, the mucosa-associated gonococci were re-isolated by cotton swaps and serial dilutions were plated on GC agar cam/erm. The colonies were enumerated after 20 h of incubation at 37°C, 5% CO_2.

Scanning Electron Microscopy and Immunohistochemistry of Vaginal Tissue

The genital tract of animals, infected for 24 h or uninfected, was excised and the longitudinally opened vaginal and uteral tissue was mounted. The whole mount organ was fixed with 2% glutaraldehyde, 3% formaldehyde in 0.1 M sodium-cacodylatebuffer, pH 6.9 containing 0.09 M sucrose, 0.01 M CaCl2, 0.01 M MgCl2 for at least 1h at 4°C. The fixed samples were further processed and analysed by scanning electron microscopy essentially as described previously (Muenzner et al., 2010). For immunohistochemistry, tissue samples were immediately fixed with 4% paraformaldehyde for at least 24 h and transferred to 10% sucrose, 0.05% cacodylic acid for 1 h at 4°C. Next, samples were transferred to a sucrose-gradient from 10 to 30% at 4°C over night. Organs were mounted in embedding medium (Cryo-M-Bed; Bright Instrument, Huntingdon, UK) and frozen at -20°C. 10 μm thick sections were cut at -20°C using a cryostat (Vacutom HM500, Microm, Germany). Sections were incubated with a mouse monoclonal antibody against CEA (clone COL-1) or a rat monoclonal antibody against murine CD105 (clone MJ7/18) together with a polyclonal rabbit antibody against *N. gonorrhoeae* MS11 (IG-511). Detection of the primary antibodies was accomplished by incubation with a combination of Cy5-conjugated goat-anti-rabbit antibody (1:250) and rhodamine-conjugated goat-anti-rat antibody (1:250; in the case of CD105 detection) or Cy3-conjugated goat-anti-mouse antibody (1:250; in the case of CEA detection). Cell nuclei were visualized by the addition of Hoechst 33342 in the final staining step. Samples were analysed with a TCS SP5 confocal laser scanning microscope (Leica, Mannheim, Germany). Images were digitally processed with Photoshop CS (Adobe Systems, Mountain View, CA) and images of individual detection chanels were merged to yield pseudo-coloured images

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Methods

For the statistical evaluation of *in vivo* infection experiments, the number of re-isolated bacteria from individual animals was compared between different treatments using the non-parametric Mann Whitney-U test or the Kruskal-Wallis One Way Analysis of Variance on Ranks as the data did not exhibit a normal distribution. Significant differences between groups are indicated by * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

DATA AND CODE AVAILABILITY

This study did not generate/analyze datasets/codes.