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Innate Recognition by Neutrophil Granulocytes Differs between *Neisseria gonorrhoeae* Strains Causing Local or Disseminating Infections

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Members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family serve as cellular receptors for *Neisseria gonorrhoeae*. More specifically, neisserial colony opacity (Opa_{CEA}) proteins bind to epithelial CEACAMs (CEACAM1, CEA, CEACAM6) to promote bacterial colonization of the mucosa. In contrast, recognition by CEACAM3, expressed by human granulocytes, results in uptake and destruction of Opa_{CEA}-expressing bacteria. Therefore, CEACAM3-mediated uptake might limit the spread of gonococci. However, some strains can cause disseminating gonococcal infections (DGIs), and it is currently unknown how these strains escape detection by granulocyte CEACAM3. Therefore, the *opa* gene loci from *N. gonorrhoeae* strain VP1, which was derived from a patient with disseminated gonococcal disease, were cloned and constitutively expressed in *Escherichia coli*. Similar to Opa proteins of the nondisseminating strain MS11, the majority of Opa proteins from strain VP1 bound epithelial CEACAMs and promoted CEACAM-initiated responses by epithelial cells. In sharp contrast to the Opa proteins of strain MS11, the Opa proteins of strain VP1 failed to interact with the human granulocyte receptor CEACAM3. Accordingly, bacteria expressing VP1 Opa proteins were not taken up by primary human granulocytes and did not trigger a strong oxidative burst. Analysis of Opa variants from four additional clinical DGI isolates again demonstrated a lack of CEACAM3 binding. In summary, our results reveal that particular *N. gonorrhoeae* strains express an Opa protein repertoire allowing engagement of epithelial CEACAMs for successful mucosal colonization, while avoiding recognition and elimination via CEACAM3-mediated phagocytosis. A failure of CEACAM3-mediated innate immune detection might be linked to the ability of gonococci to cause disseminated infections.

Gonorrhea is one of the most common sexually transmitted infections on a global scale. The causative agent of gonorrhea is the human-restricted, Gram-negative bacterium *Neisseria gonorrhoeae*. In a large proportion of infected people, particularly women, the infection is asymptomatic. Furthermore, in most symptomatic patients, the bacteria are limited to the lower urogenital tract, with a characteristic purulent discharge containing a high load of granulocytes as a hallmark of the infection. Whereas this kind of limited local inflammation can resolve even without treatment, ascending infections of the upper genital tract can lead to complicated disease manifestations such as pelvic inflammatory disease, a serious risk factor for female infertility and ectopic pregnancy. Moreover, certain gonococcal strains are able to access and survive in human blood, causing systemic or disseminated gonococcal infections (DGIs) with often severe consequences, including arthritis, bacterial endocarditis, or meningitis.

The ability of certain gonococcal strains to cause disseminated disease has been attributed to specific traits more often associated with strains isolated from DGI patients than strains from patients with local infections. One feature frequently reported for DGI-causing strains is the phenotype of auxotrophy for arginine, hypoxanthine, and uracil (AHU phenotype) (1). However, this group of strains seems to represent a clonal lineage which appears to be absent in some parts of the world (2, 3). A more general characteristic of DGI strains is the prevalence of a particular outer membrane porin isoform, $PorB_{IA}$, which differs from the other existing isoform, $PorB_{IB}$, found in the majority of isolates from localized infections (4). The $PorB_{IA}$-defined serogroup is present in virtually all AHU phenotype strains and in about 60% of the non-AHU phenotype strains isolated from DGI patients (4, 5). The ability of $PorB_{IA}$ to mediate serum resistance by binding to complement factor H and C4bBP (6, 7) as well as its ability to promote host cell invasion under low-phosphate conditions (8, 9) might contribute to the prevalence of this outer membrane protein variant in DGI strains. Interestingly, a large fraction (35 to 40%) of the non-AHU phenotype gonococcal strains isolated from DGI patients harbor the more prevalent $PorB_{IB}$ isoform (4). Therefore, alternative traits might also predispose gonococci to cause disseminated forms of disease.

In addition to humoral factors, such as the complement system or antigonococcal antibodies, cell-based innate immune mechanisms are thought to limit gonococcal infections. Granulocytes are massively recruited to the sites of infection and are able to recognize and eliminate gonococci in an opsonin-independent manner (10). A key host factor mediating rapid phagocytosis and destruction of gonococci is carcinoembryonic antigen-related cell

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adhesion molecule 3 (CEACAM3), a granulocyte membrane glycoprotein (11, 12). Recognition by CEACAM3 depends on the presence of particular colony opacity (Opa) protein variants (13). Up to 12 distinct *opa* gene loci are present in the genome of a single *N. gonorrhoeae* strain. Each locus appears to be constitutively transcribed, but expression of individual Opa proteins is independently regulated on the translational level. This phase variation is due to several pentameric repeat units in the 5′ coding region of each *opa* gene, and these repeat units determine the reading frame (14). Addition or deletion of pentameric repeats during replication of the bacterial chromosome, most likely due to slipped-strand mispairing, corrects or compromises the reading frame and arrests individual Opa proteins in an on or an off phase (15).

Opa proteins serve as important adhesins which mediate intimate attachment of *N. gonorrhoeae* to host epithelial cells (16). Functional analysis of the complete Opa protein repertoire of gonococcal strain MS11 has demonstrated that 1 out of the 11 Opa proteins can bind to heparan sulfate proteoglycans (HSPGs) or, via recruitment of vitronectin and fibronectin, to host cell integrins (17–19). To indicate their particular binding specificity, Opa proteins binding to HSPGs have also been termed Opa_{HSPG} (16). The other 10 Opa proteins of MS11 associate with one or several members of the CEACAM family, in particular, CEACAM1, CEACAM3, CEA, and CEACAM6 (20, 21), and, accordingly, have been designated Opa_{CEA} (16). In this respect, gonococcal Opa_{CEA} proteins bind only to human CEACAM family members and not to CEACAM orthologues from other species (22). By engaging CEACAM1, CEA, or CEACAM6 on the apical surface of epithelial cells (epithelial CEACAMs), gonococci seem to profit during mucosal colonization (23). In particular, attachment of bacteria to epithelial CEACAMs triggers enhanced integrin-mediated host cell adhesion to the extracellular matrix and counteracts the shedding of infected epithelial cells from the tissue (24, 25). On the other hand, recognition of Opa_{CEA} proteins by CEACAM3 might limit the spread of gonococci due to granulocyte-mediated opsonin-independent phagocytosis. Indeed, the genome of strain MS11, which is associated with localized infections (26, 27), encodes several CEACAM3-binding Opa_{CEA} proteins (20, 21). It is conceivable that the CEACAM-binding profile and, in particular, the lack of CEACAM3-binding Opa proteins might contribute to the ability of certain gonococcal strains to evade opsonin-independent recognition by granulocytes and to cause disseminated disease. However, the redundancy of *opa* genes in the gonococcal genome and the frequent phase variation of Opa protein expression have impaired a comprehensive analysis of CEACAM-binding Opa proteins.

Therefore, the present study was initiated to determine the CEACAM-binding profile of the Opa protein repertoire of a DGI strain. Accordingly, we cloned all Opa genes of strain VP1, a clinical isolate from a patient with disseminated gonorrhea (28). The reading frame of all VP1 Opa proteins was arrested in the on phase to circumvent phase variation, and individual Opa proteins were heterologously expressed in *Escherichia coli*. Similar to the Opa proteins of MS11, most VP1 Opa proteins bound to human CEACAMs. In line with the ability of VP1 to engage epithelial CEACAMs, VP1 Opa_{CEA} proteins were able to trigger enhanced host cell adhesion to the extracellular matrix. In contrast, VP1 Opa_{CEA} proteins did not bind CEACAM3 and did not trigger the opsonin-independent uptake and destruction by primary human granulocytes. Analysis of Opa variants from four additional clinical DGI isolates further strengthened the hypothesis that DGI strains can evade CEACAM3-dependent recognition and subsequent elimination by innate immune cells and that a lack of CEACAM3-binding Opa proteins might predispose gonococcal strains to cause disseminated infections.

MATERIALS AND METHODS

Cell culture and transfection. Cells of the human embryonic kidney cell line 293T (293 cells) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum at 37°C in 5% CO_2 and subcultured every 2 to 3 days. 293 cells were transfected by calcium phosphate coprecipitation using 5 to 8 μg of plasmid DNA for each 10-cm culture dish. Primary human granulocytes were isolated from peripheral blood as described previously and used immediately for infection experiments (13).

Neisserial strains and growth conditions. Opa-expressing, nonpiliated *N. gonorrhoeae* MS11-B2.1 (strains N303 [Opa_{50}], N304 [Opa_{53}], N305 [Opa_{51}], N306 [Opa_{59}], N307 [Opa_{55}], N308 [Opa_{56}], N309 [Opa_{52}], N310 [Opa_{60}], N311 [Opa_{54}], N312 [Opa_{58}], N313 [Opa_{57}] expressing the Opa proteins indicated in brackets), nonpiliated, nonopaque gonococcus MS11-B2.1 (strain N302), nonpiliated MS11-F3 expressing the VP1-Opa68 protein (strain N554), and wild-type strain *N. gonorrhoeae* VP1 isolated from a patient with DGI (28, 29) were kindly provided by T. F. Meyer (Max-Planck Institut für Infektionsbiologie, Berlin, Germany). Whereas strain MS11 expresses $PorB_{IB}$, strain VP1 expresses $PorB_{IA}$ (8). Gonococcal strains 11, 102, 229, and 241 were isolated from blood samples from four different patients with DGIs. All those isolates were low-passage-number clinical strains and had the porin $PorB_{IA}$ isoform; however, they were all of different serological variants. Neisseriae were grown on GC agar plates (Difco BRL, Paisley, United Kingdom) supplemented with appropriate antibiotics and growth supplement at 37°C in 5% CO_2 and subcultured daily. For infection or pulldown assays, bacteria grown overnight were taken from GC agar plates and suspended in phosphate-buffered saline (PBS), and the numbers of CFU were estimated from readings of the optical density at 550 nm according to a standard curve.

Opa protein nomenclature. Due to the large number of *opa* gene alleles, the Opa protein nomenclature is confusing. In this paper, we have utilized and extended the Opa protein nomenclature introduced by Kupsch et al. (30). In this respect, the 11 cloned *opa* genes from *N. gonorrhoeae* strain MS11, which are fixed in the on phase by silent mutations in the leader peptide coding sequence and which are either expressed from a plasmid in *N. gonorrhoeae* or heterologously expressed in *E. coli*, are numbered Opa_{50} to Opa_{60} according to the increasing apparent molecular weight of the encoded Opa protein upon SDS-PAGE (30). In a similar manner, the four previously cloned *opa* genes from *N. gonorrhoeae* strain VP1 were numbered Opa_{65} to Opa_{68} (30). We have cloned seven additional *opa* genes from *N. gonorrhoeae* VP1 and named them consecutively Opa_{69} to Opa_{75}. Furthermore, to indicate the specific binding capacity and the functionality of the Opa proteins, we have extended the nomenclature of Hauck and Meyer (16), who distinguished between Opa_{HSPG} and Opa_{CEA}. It has become clear that most Opa_{CEA} proteins exclusively bind to one or all CEACAMs present on epithelial cells (i.e., CEACAM1, CEA, or CEACAM6). Accordingly, we have termed these Opa proteins $Opa_{CEA\text{-}e}$ (where the "e" stands for "epithelial CEACAMs"). In contrast, only a minority of Opa_{CEA} proteins binds to epithelial CEACAMs as well as the granulocyte-restricted CEACAM3. Therefore, we have designated these proteins $Opa_{CEA\text{-}a}$ (where the "a" stands for "all CEACAMs").

Expression of Opa proteins in *Escherichia coli*. The MS11 Opa_{52}-expressing *E. coli* strain was described previously (30) and was kindly provided by T. F. Meyer (Max-Planck Institut für Infektionsbiologie, Berlin, Germany). In order to express VP1 Opa proteins, *opa* genes were amplified by PCR from chromosomal DNA of wild-type strain *N. gonorrhoeae* VP1 with the primers Opa-XhoI-sense (5′-CCTCTCGAGTCTCT TCTCTTCTCTTCC-3′) and Opa1/2-MC58-HindIII-anti (5′-GGTCAA AGCTTTCAGAAGCGGTAGCG-3′) and cloned in pCR Blunt II-TOPO

(Invitrogen). To suppress phase variation, the cloning strategy from Kuespert et al. was followed (31). Accordingly, opa_{65}, opa_{67}, opa_{69}, opa_{70}, opa_{71}, opa_{72}, opa_{73}, opa_{74}, and opa_{75} were amplified using the primers Opa-MC58-mitte-sense (5′-ATCGCTTCTATTTAGCTCTTTATTGT TCAGTTCCCTACTCTTCAGCTCCGCAGCGCAGGCGGCAACTGA-3′) and Opa1/2-MC58-HindIII-anti. The PCR product served as a template for a second PCR using the primers OpaMC58 (pET28)-NcoI-sense (5′-GGCGCCCATGGAACCAGCCCCCAAAAAACCTTCTCTCCTGT TCTCATCGCTTCTATTTAGCTCTTTA-3′) and Opa1/2-MC58-HindIII-anti. The products of the second PCR were digested with NcoI and HindIII and cloned into pET-28a (Novagen). VP1 opa_{68} was amplified by PCR from DNA of *N. gonorrhoeae* strain N554 and cloned into pET-28a. The pET-28a vectors encoding Opa proteins were verified by sequencing and transformed into *E. coli* BL21(DE3) (Novagen), which was induced for protein expression by IPTG (isopropyl-β-D-thiogalactopyranoside). All *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics.

Recombinant plasmid constructs. Mammalian expression plasmids encoding the soluble green fluorescent protein (GFP)-tagged amino-terminal domain of CEACAM1, CEACAM3, CEA, or CEACAM6 were described previously (32). Mammalian expression vectors encoding GFP-tagged or mKate-tagged full-length CEACAM1-4L (the 4L isoform) or full-length CEACAM3 were described previously (33–35). A mammalian expression vector encoding GFP-tagged CD105 was also described previously (24).

Cell lysis and Western blotting. Cell lysis and Western blotting were performed as described earlier (36) using a monoclonal antibody against GFP (clone JL-8; Clontech) or a monoclonal antibody against Opa proteins (clone 4B12/C11; a generous gift of Mark Achtman, University College Cork, Cork, Ireland). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Determination of CEACAM binding by different bacteria. Expression of the soluble N-terminal domains of human CEACAMs in 293 cells and binding studies with different pathogens were performed as described previously (32). Briefly, 2×10^7 bacteria were added to CEACAM N domain-containing cell culture supernatant in a total volume of 1 ml and incubated for 1 h. After incubation, the bacteria were washed twice with PBS and either boiled in SDS sample buffer prior to SDS-PAGE and Western blotting or taken up in PBS–2% fetal calf serum (flow buffer) and analyzed by flow cytometry.

Gentamicin protection assay. Gentamicin protection assays were conducted as described previously (13). Briefly, 293 cells were transfected with CEACAM-encoding or control plasmids by standard calcium phosphate precipitation. At 1 day posttransfection, 5×10^5 cells were seeded in 24-well plates coated with gelatin. On the next day, cells were infected with 100 bacteria/cell (multiplicity of infection [MOI], 100), and 1 h later, the medium was replaced with DMEM containing 100 μg/ml gentamicin to kill extracellular bacteria. After 45 min of incubation in gentamicin-containing medium, cells were lysed by the addition of 1% saponin in PBS for 15 min. Suitable dilutions were plated in triplicate on GC agar to determine the number of recovered intracellular bacteria.

Cell adhesion assay. Cell adhesion to extracellular matrix proteins was measured essentially as described previously (25). Briefly, the wells of 96-well plates were coated with 100 μl PBS containing the indicated concentrations of collagen type 1 from calf skin (ICN Biomedicals, Irvine, CA) or bovine serum albumin (BSA) for 24 h at 4°C. After coating, the wells were blocked with 0.2% BSA in PBS for 1 h at room temperature. 293 cells were transfected with pLPS3′EGFP-CD105 (CD105-GFP) or pcDNA-CEACAM1, and at 1 day after transfection, the cells were serum starved for 16 h. Serum-starved cells were infected with the indicated bacteria at an MOI of 30 for 8 h. Following infection, the cells were detached by limited trypsin-EDTA treatment, which was stopped by addition of soybean trypsin inhibitor (0.5 mg/ml in DMEM). Detached cells were kept suspended in suspension medium (DMEM, 0.2% BSA) for 1 h at 37°C and replated at 4×10^4 cells/well in the protein-coated wells of the 96-well plate. Cells were allowed to adhere for 90 min at 37°C, before nonadherent cells were removed by gentle washing with PBS. Adherent cells were fixed and stained with 0.1% crystal violet in 0.1 M borate, pH 9, for 60 min. After washing and drying, the crystal violet was eluted in 10 mM acetic acid and the staining intensity was measured at 550 nm in a Varioskan Flash microplate reader (Thermo Fisher Scientific, Germany).

Immunofluorescence staining. Primary human granulocytes were resuspended in phagocytosis buffer (PB; 1× PBS, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 1% heat-inactivated calf serum), seeded in 24-well plates, and incubated for 30 min at 37°C. The granulocytes were infected (MOI, 40) with the different *E. coli* strains for 30 min at 37°C. Samples were washed 3 times with PBS, fixed with 4% paraformaldehyde in PBS, and again washed 3 times with PBS prior to incubation in blocking buffer (PBS, 10% fetal calf serum) for 20 min. Samples were stained with polyclonal anti-*E. coli* lipopolysaccharide (LPS) antibody (diluted 1:200 in blocking buffer; AbD Serotec) for 1 h. After 3 washes and 5 min of incubation in blocking buffer, samples were incubated with Cy2-coupled goat antirabbit antibodies (1:200) in blocking buffer for 45 min. Following 3 washes, samples were permeabilized with 0.2% saponin in PBS for 10 min, washed 3 times, and again blocked with blocking solution. Samples were stained with polyclonal anti-*E. coli* LPS antibody and Cy5-coupled goat antirabbit antibody as the secondary antibody, resulting in Cy2/Cy5-labeled extracellular and exclusively Cy5-labeled intracellular bacteria. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000) for 10 min. The samples were embedded in mounting medium (Dako, Glostrup, Denmark) and viewed with a TCS SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). Fluorescence signals of triple-labeled specimens were serially recorded with appropriate excitation and emission filters to avoid bleed-through. Images were digitally processed with NIH ImageJ software and merged to yield pseudocolored pictures.

Oxidative burst measurements. Granulocytes (2×10^5) were suspended in 200 μl chemiluminescence buffer (8 g/liter NaCl, 0.2 g/liter KCl, 0.62 g/liter KH_2PO_4, 1.14 g/liter Na_2HPO_4, 1 g/liter glucose, 50 mg/liter BSA, pH 7.2) containing luminol (20 μg/ml). Granulocytes were transferred to a 96-well plate and infected with 1×10^7 bacteria or left uninfected. Phorbol myristate acetate (1 μg/ml) was used as a positive control for oxidative burst. Chemiluminescence was determined every 2 min at 37°C with a Varioskan Flash reader. To determine the total amount of reactive oxygen produced, the response curves were exported to GraphPad Prism software, and the areas under the curves over 90 min were calculated.

Nucleotide sequences accession numbers. The nucleotide sequences of VP1 Opa_{69} to Opa_{75} were deposited in GenBank under accession numbers KC503485 to KC503491.

RESULTS

***N. gonorrhoeae* MS11 Opa proteins bind to soluble domains of human CEACAMs.** The present study was initiated with the aim to compare the CEACAM-binding properties of *N. gonorrhoeae* MS11, which causes local infections, with those of the disseminating strain VP1. Accordingly, the 11 genetically defined Opa proteins of the *N. gonorrhoeae* MS11 strain were analyzed regarding their CEACAM-binding properties. First, Opa protein expression of all strains was verified by Western blotting with a monoclonal antibody against neisserial Opa proteins (Fig. 1A). Furthermore, the soluble domains of the indicated CEACAMs were expressed in 293 cells, and the recombinant CEACAM domains were adjusted to the same concentration (Fig. 1B). To test CEACAM binding, recombinant *N. gonorrhoeae* MS11 strains lacking Opa protein expression or constitutively expressing Opa_{50} to Opa_{60} were incubated with the GFP-tagged soluble amino-terminal domains of CEACAM1, CEA, CEACAM3, and CEACAM6 and washed extensively, and the bacterium-associated receptor domains were detected by Western blotting. Upon incubation with the recombi-

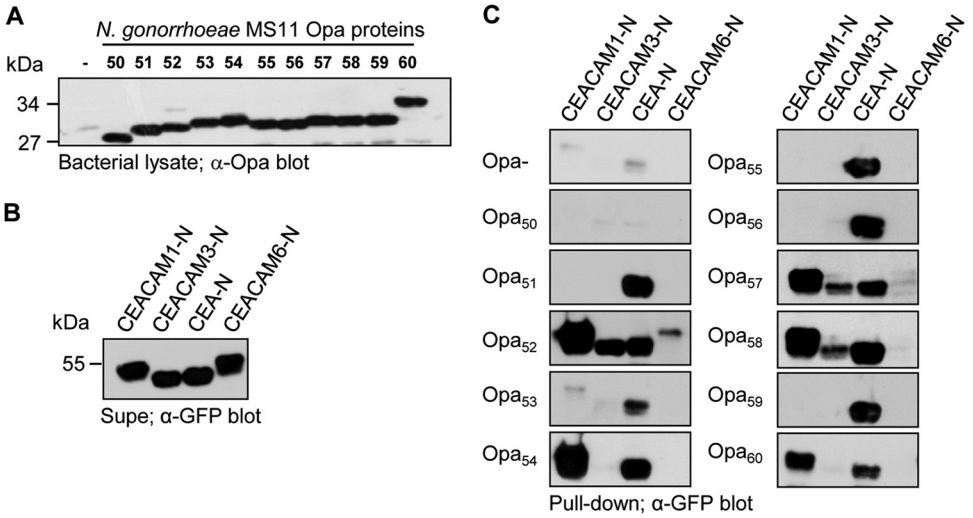

FIG 1 Opa proteins of *N. gonorrhoeae* strain MS11 bind to amino-terminal domains of CEACAM1, CEACAM3, CEA, and CEACAM6. (A) *N. gonorrhoeae* MS11 strains lacking Opa protein expression (lane −) or expressing a single defined Opa protein (Opa_{50} to Opa_{60} [lanes 50 to 60, respectively]) were lysed, and Opa protein expression was verified by Western blotting using a monoclonal anti-Opa protein antibody (clone 4B12/C11). (B) Cell culture supernatants (Supe) containing the amino-terminal domains of the indicated human CEACAMs fused to GFP were collected, and the amount of the GFP fusion proteins was analyzed by Western blotting with a monoclonal anti-GFP antibody. (C) The soluble CEACAM-GFP fusion proteins were used in pulldown assays together with *N. gonorrhoeae* MS11 strains expressing the Opa proteins indicated on the left side of the blots. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs coprecipitating with the bacteria.

nant proteins, Opa-negative variants of MS11 did not bind to any CEACAM (Fig. 1C). Importantly, with the exception of Opa_{50}-expressing bacteria (which are known to bind to heparan sulfate proteoglycans; Opa_{HSPG}), each Opa protein expressed by the MS11 strain was able to bind at least one of the recombinant CEACAM domains (Fig. 1C). The interactions identified with the soluble recombinant proteins were in agreement with those found in previous studies where the binding of *N. gonorrhoeae* MS11 Opa proteins (expressed in *E. coli* or *N. gonorrhoeae*) to CEACAM-expressing HeLa cell lines was analyzed (20, 21). From the binding profiles, it became apparent that CEACAM-recognizing Opa proteins (Opa_{CEA} proteins) of strain MS11 can be grouped into two categories. The first is a large group of Opa_{CEA} proteins which exclusively binds to CEACAMs found on epithelial cells. Opa proteins with such a binding profile, e.g., Opa_{51}, Opa_{53}, Opa_{54}, Opa_{55}, Opa_{56}, Opa_{59}, and Opa_{60}, are designated $Opa_{CEA\text{-}e}$ (where the "e" stands for "epithelial CEACAMs"). In contrast, a small group of MS11 Opa_{CEA} proteins (comprising Opa_{52}, Opa_{57}, and Opa_{58}) binds to epithelial CEACAMs as well as to the granulocyte-restricted CEACAM3. Therefore, these proteins are designated $Opa_{CEA\text{-}a}$ (where the "a" stands for "all CEACAMs").

***N. gonorrhoeae* VP1 contains 11 distinct Opa proteins.** In contrast to the 11 distinct Opa proteins encoded by the genome of *N. gonorrhoeae* MS11 (37), only 4 Opa proteins have been described and partially characterized so far from the *N. gonorrhoeae* strain VP1 (30, 38). To identify the complete repertoire of VP1 *opa* genes, VP1 chromosomal DNA was isolated and a PCR with primers targeting the conserved regions of *opa* genes was performed. Subsequently, the *opa* amplicons were ligated into pCR Blunt II-TOPO, and single clones from this *opa* amplicon library were sequenced to identify the respective *opa* genes. Using this strategy, we were able to identify nine different VP1 *opa* genes. Whereas two of these nine sequences (Opa_{65} and Opa_{67}) had been described in the study by Kupsch et al. (30), seven VP1 *opa* genes appear to be novel sequences not reported previously. Further sequencing of more than 80 clones from the VP1 *opa* amplicon library did not retrieve the previously described opa_{66} or opa_{68} or any additional new *opa* sequence. For our further study, the cloned opa_{68} gene was kindly provided by T. F. Meyer (Max-Planck Institut für Infektionsbiologie, Berlin, Germany), but the previously reported opa_{66} clone had been lost from that strain collection. Therefore, we established a further VP1 *opa* amplicon library and screened about 100 additional clones with opa_{66}-specific primers, but we were not able to detect the opa_{66} sequence among these cloned VP1 *opa* sequences. The seven novel *opa* loci were named consecutively opa_{69} to opa_{75} with regard to the 4 VP1 Opa proteins already described (Opa_{65}, Opa_{66}, Opa_{67}, Opa_{68}), implicating that the genome of this strain encodes a total of 11 Opa proteins. The deduced amino acid sequences of all VP1 Opa proteins were compared by multiple-sequence alignment (Fig. 2). All Opa proteins showed the known domain architecture with conserved regions, the semivariable (SV) region, the hypervariable 1 (HV-1) region, and the HV-2 region, which are proposed to be responsible for receptor binding (Fig. 2). Interestingly, the amino acid sequence of Opa_{65}, which we have cloned, differed from the previously reported sequence of Opa_{65} at eight positions, mainly in the leader peptide (amino acid positions 3, 19, 21, 28, 35, 37, and 38) and at amino acid position 129, harboring a phenylalanine residue (which seems to be conserved in all VP1 Opa sequences) instead of the previously reported isoleucine residue (30). Therefore, the mature, membrane-embedded Opa_{65} protein is almost identical between the two studies.

***N. gonorrhoeae* VP1 Opa proteins bind to soluble domains of epithelial CEACAMs but not to soluble domains of granulocyte receptor CEACAM3.** Individual VP1 *opa* genes were then amplified by an established PCR strategy which abolishes the pentameric repeats contained within the leader peptide coding sequence and arrests the gene in the correct reading frame (30, 31). The

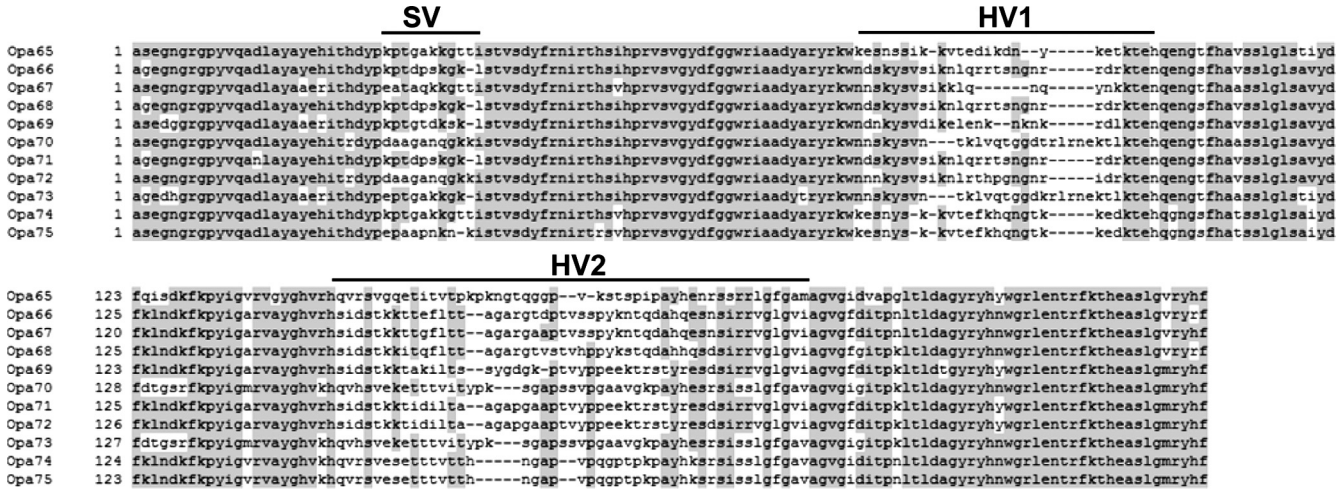

FIG 2 Amino acid sequence alignment of *N. gonorrhoeae* VP1 Opa proteins. VP1 Opa protein sequences were aligned by pairwise alignment using Clone manager software. The SV, HV-1, and HV-2 regions are indicated. Identical amino acids are shaded. Deleted amino acids are indicated by dashes. The corresponding nucleotide sequences were deposited in GenBank under accession numbers KC503485 to KC503491.

on-phase-arrested VP1 *opa* genes were subcloned into the expression vector pET-28a under the control of the T7 promoter, and the plasmids were transformed in *E. coli* BL21(DE3). Upon IPTG induction, all cloned VP1 Opa proteins were successfully expressed in *E. coli* (Fig. 3A). To analyze the CEACAM-binding profile of the 10 different VP1 Opa proteins, we incubated the bacteria with similar amounts of soluble GFP-fused CEACAM1, CEA, CEACAM3, and CEACAM6 amino-terminal domains (Fig. 3B). After washing, the bacterium-associated GFP fluorescence was detected by flow cytometry as a measure of the interaction between individual VP1 Opa proteins and human CEACAMs. As a positive control, *E. coli* expressing the Opa_{52} protein of *N. gonorrhoeae* MS11 was employed and showed clear binding to all four distinct CEACAMs (Fig. 3C). The flow cytometry results with the MS11 Opa_{CEA-a} protein (Opa_{52}) expressing *E. coli* corroborate the findings obtained by Western blotting with Opa_{52}-expressing *N. gonorrhoeae* (Fig. 1C). Importantly, most VP1 Opa proteins expressed in *E. coli* demonstrated clear binding to the amino-terminal domains of CEACAM1 or CEA. None of these Opa proteins was, however, associated with CEACAM3 in a comparable manner (Fig. 3C). There was a very weak binding to CEACAM3 observed for a single VP1 Opa protein, as *E. coli* expressing the VP1 Opa_{70} protein showed a slight shift in GFP fluorescence upon incubation with the soluble CEACAM3 amino-terminal domain (Fig. 3C). The results of these binding studies are summarized in Table 1. Together, Opa_{CEA} proteins of *N. gonorrhoeae* strain VP1 selectively bind to epithelial CEACAMs, but none of these Opa_{CEA} proteins is a clear-cut ligand for the granulocyte receptor CEACAM3. These results would imply that the genome of the disseminating gonococcal strain encodes Opa_{CEA-e} proteins but no Opa_{CEA-a} variants and might not trigger opsonin-independent granulocyte responses.

CEACAM1, but not CEACAM3, mediates uptake of *E. coli* VP1 Opa_{65}. Previous studies have already demonstrated that CEACAM interaction with MS11 Opa_{CEA} proteins can lead to bacterial internalization into eukaryotic cells (20, 39, 40). To address if VP1 Opa_{CEA} proteins can also initiate CEACAM-mediated uptake, we employed gentamicin protection assays. To better compare MS11 Opa proteins and VP1 Opa proteins, we employed them in the same heterologous strain background in *E. coli*. Furthermore, we chose *E. coli* VP1 Opa_{65} as a prototypical Opa_{CEA-e} protein of this strain. 293 cells were transfected with GFP, GFP-tagged CEACAM1, or GFP-tagged CEACAM3, and the expression of the proteins was verified by Western blotting (Fig. 4A). Next, cells were infected for 1 h with *E. coli* expressing VP1 Opa_{65}, *E. coli* expressing MS11 Opa_{52}, or an *E. coli* control strain lacking Opa protein expression (Fig. 4B). Following the infection, extracellular bacteria were killed by gentamicin and the amount of viable, intracellular bacteria was determined. As expected, *E. coli* Opa_{52} was taken up by CEACAM1- as well as CEACAM3-expressing cells (Fig. 4C). Similarly, *E. coli* Opa_{65} was internalized by CEACAM1-expressing cells. However, CEACAM3 did not mediate uptake of *E. coli* Opa_{65} (Fig. 4C). This is in line with the results obtained with soluble CEACAM domains, indicating that VP1 Opa_{65} shows selective binding to CEACAM1 but does not interact with CEACAM3. In summary, these data support the idea that the Opa_{CEA} proteins of *N. gonorrhoeae* VP1 selectively bind to epithelial CEACAMs and circumvent binding to the granulocyte receptor CEACAM3.

***E. coli* VP1 Opa_{65} binding to CEACAM1 leads to CD105 upregulation and increased cell adhesion.** On the basis of previous studies with CEACAM-binding bacteria, it can be postulated that Opa_{CEA-e} proteins should promote bacterial colonization of the mucosa by suppressing epithelial exfoliation (25). The mechanistic basis of this process is the CEACAM-triggered *de novo* expression of CD105, which in turn enhances integrin-mediated matrix adhesion of the infected host cells (24). To test if VP1 Opa_{CEA-e} proteins can initiate CD105 upregulation and enhance matrix adhesion of human cells, 293 cells were transiently transfected with mKate-tagged CEACAM1 (Fig. 5A). Next, CEACAM1-mKate-expressing 293 cells were infected for 6 h with *E. coli* VP1 Opa_{65}, *E. coli* MS11 Opa_{52}, or *E. coli* lacking Opa protein expression. Following infection, cells were analyzed for CD105 expression by flow cytometry. Uninfected cells or cells infected with *E. coli* lacking Opa protein expression exhibited low background staining for CD105, suggesting that 293 cells have a very low level of CD105

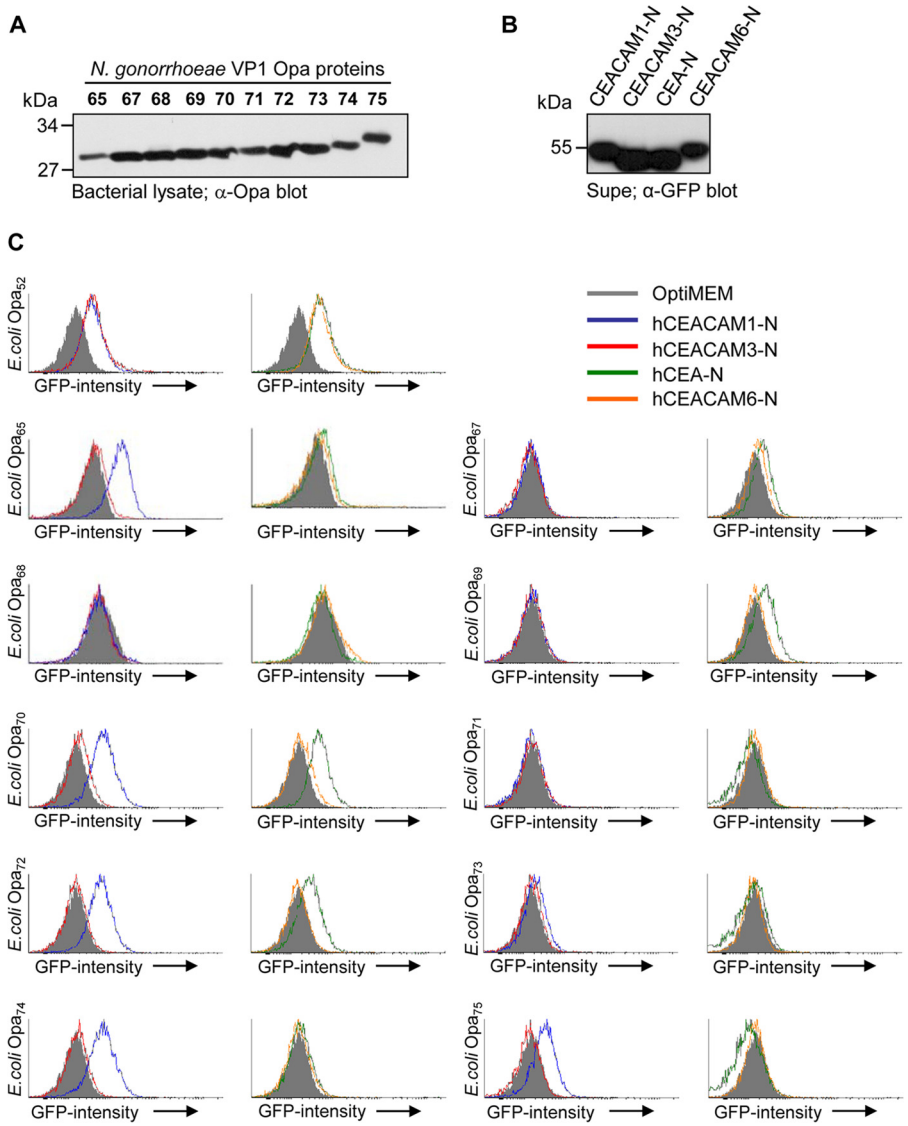

FIG 3 *N. gonorrhoeae* VP1 Opa proteins interact with amino-terminal domains of CEACAM1 and CEA, but not with CEACAM3. (A) The expression of VP1 Opa proteins in *E. coli* was verified by Western blotting with a monoclonal anti-Opa antibody. (B) The expression of GFP-tagged soluble CEACAM domains was determined by Western blotting of culture supernatants with a monoclonal anti-GFP antibody. (C) Supernatants containing GFP-tagged, soluble domains of the indicated CEACAMs were incubated with VP1 Opa protein-expressing *E. coli*. After washing, bacteria were analyzed by flow cytometry and the bacterium-associated GFP fluorescence was determined as a measure of the interaction between individual VP1 Opa proteins and human CEACAMs. In each case, 10,000 events were determined.

expression (Fig. 5B). Clearly, upon infection with *E. coli* Opa_{65} or *E. coli* Opa_{52}, an increased surface expression of CD105 could be observed (Fig. 5B). To analyze if CEACAM1-triggered CD105 expression has functional consequences with regard to host cell adhesion to the extracellular matrix, infected cells were employed in matrix adhesion assays. First, 293 cells were transiently transfected either with plasmids encoding CEACAM1-GFP or, as a positive control, with CD105-GFP. Expression of the constructs by the transfected cells was verified by Western blotting (Fig. 5C). As observed before, adhesion of CD105-GFP-expressing cells to the extracellular matrix protein collagen, an integrin β1 ligand, was strongly increased compared to that of control cells (Fig. 5D). Next, CEACAM1-GFP-transfected 293 cells were infected for 12 h with the indicated *E. coli* strains, and then their matrix adhesion was determined. Importantly, CEACAM1 engagement by *E. coli* Opa_{52} or *E. coli* Opa_{65} increased the adhesiveness of infected cells to collagen (Fig. 5E). In contrast, *E. coli* lacking Opa protein expression was not able to enhance cell matrix adhesion (Fig. 5E). Taken together, these results suggest that Opa_{CEA-e} proteins of *N. gonorrhoeae* VP1 can exploit epithelial CEACAMs to modulate host cell adhesion, a process which could contribute to successful mucosal colonization.

***N. gonorrhoeae* VP1 Opa_{CEA-e} proteins fail to trigger a strong oxidative burst response by human granulocytes.** The selective association of VP1 Opa_{CEA} proteins with epithelial CEACAMs and the failure of VP1 Opa_{CEA} proteins to engage CEACAM3 suggest that this strain is able to escape opsonin-independent recognition by human granulocytes. To investigate if *N. gonorrhoeae*

TABLE 1 CEACAM- and HSPG-binding profile of *N. gonorrhoeae* strain MS11 and strain VP1 Opa proteins

Receptor	Binding[a]																					
	MS11										VP1											
	Opa_{50}	Opa_{51}	**Opa_{52}**	Opa_{53}	Opa_{54}	Opa_{55}	Opa_{56}	**Opa_{57}**	**Opa_{58}**	Opa_{59}	Opa_{60}	Opa_{65}	Opa_{66}[b]	Opa_{67}	Opa_{68}	Opa_{69}	Opa_{70}	Opa_{71}	Opa_{72}	Opa_{73}	Opa_{74}	Opa_{75}
HSPG	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	ND	ND	ND	ND	ND	ND	ND
CEACAM1	–	–	+++	–	+++	–	–	+++	+++	–	+++	+++	ND	–	–	–	+++	–	+++	+	+++	+++
CEA	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	ND	+++	–	+++	+++	–	+++	+	–	–
CEACAM6	–	–	+++	–	–	–	–	+	+	–	–	–	ND	+	–	–	+	–	–	–	–	–
CEACAM3	–	–	+++	–	–	–	–	+++	+++	–	–	–	ND	–	–	–	+	–	–	–	–	–

[a] CEACAM-binding properties of Opa proteins derived from *N. gonorrhoeae* strain MS11 or strain VP1 determined in this study using soluble, GFP-tagged amino-terminal CEACAM domains. +++, strong binding; +, weak binding; –, no binding; ND, not determined. Opa proteins showing strong CEACAM3 binding are indicated in bold. The HSPG-binding profile of Opa proteins was analyzed in the study by Kupsch et al. (30).

[b] Opa_{66} was not analyzed in this study.

VP1 Opa_{CEA} proteins can trigger opsonin-independent phagocytosis, we infected primary human granulocytes for 30 min with *E. coli* expressing VP1 Opa_{65}, *E. coli* expressing MS11 Opa_{52}, or *E. coli* lacking Opa protein expression. Infected samples were stained to differentiate between intracellular bacteria (stained with Cy5) and extracellular bacteria (labeled with Cy2 and Cy5). As expected, *E. coli* lacking Opa protein expression did not interact with human granulocytes in the absence of opsonins (Fig. 6A). In contrast, *E. coli* Opa_{52} bound to and was internalized by the human granulocytes under these conditions (Fig. 6A). Importantly, though *E. coli* Opa_{65} was found to associate with the granulocytes (most likely due to the expression of CEACAM1 by human granulocytes), the bacteria were not phagocytosed (Fig. 6A). This is in agreement with previous results, showing that CEACAM3 engagement, but not CEACAM1 binding, by Opa_{CEA} proteins is critical for efficient opsonin-independent uptake by human granulocytes (13, 34, 41). Furthermore, binding of MS11 Opa_{CEA-a} proteins to CEACAM3 is known to trigger the production of reactive oxygen species by granulocytes and finally results in the destruction of the bacteria (34, 42). In contrast, the heparan sulfate proteoglycan binding Opa_{HSPG} or Opa_{CEA-e} proteins of strain MS11 do not trigger oxidative responses (see Fig. S1 in the supplemental material) (34). According to our analyses, we would therefore predict that the Opa_{CEA} proteins of strain VP1 are not able to induce a comparable oxidative burst. To address this question, we measured the production of reactive oxygen species by primary human granulocytes in response to *E. coli* expressing defined gonococcal Opa proteins. Clearly, an *E. coli* strain transformed with the empty expression vector did not stimulate an oxidative burst, whereas a strain harboring a CEACAM3-binding Opa_{CEA-a} protein of strain MS11 (Opa_{52}) induced a clear oxidative response during the first 1 h of bacterium-phagocyte interaction (Fig. 6B). Importantly, *E. coli* expressing the CEACAM-binding Opa_{65} protein derived from strain VP1 failed to induce the induction of reactive oxygen metabolites (Fig. 6B). We next measured the oxidative burst induced by our complete panel of *E. coli* strains expressing MS11 or VP1 Opa proteins and calculated the total amount of reactive oxygen production by calculating the area under the curve over a period of 100 min (Fig. 6C). Importantly, none of the *E. coli* strains expressing an Opa_{CEA-e} protein of *N. gonorrhoeae* VP1 triggered an oxidative response comparable to that triggered by the MS11 Opa_{52} protein (Fig. 6C). Only the *N. gonorrhoeae* VP1 Opa_{70} protein, which had shown a slight CEACAM3 association before in binding assays with the recombinant receptor (Fig. 3C), led to a significant oxidative response (Fig. 6C). The response induced by the VP1 Opa_{70} protein was dependent on the preparation and the donor of the primary granulocytes, with the maximum response producing about 50% of the total reactive oxygen produced by the *N. gonorrhoeae* MS11 Opa_{52} protein, as shown in the representative experiment (Fig. 6C). Taken together, these results demonstrate that the complete set of CEACAM-binding Opa proteins derived from the disseminating strain *N. gonorrhoeae* VP1 fails to induce strong granulocyte responses in the absence of opsonizing agents.

Multiple DGI isolates do not bind CEACAM3. To investigate if a potential escape from CEACAM3-mediated immune recognition and subsequent elimination might be a more general phenomenon, we tested four additional clinical gonococcal strains, derived from blood cultures of DGI patients, for their CEACAM3-binding capacity. First, opaque colonies from these DGI strains

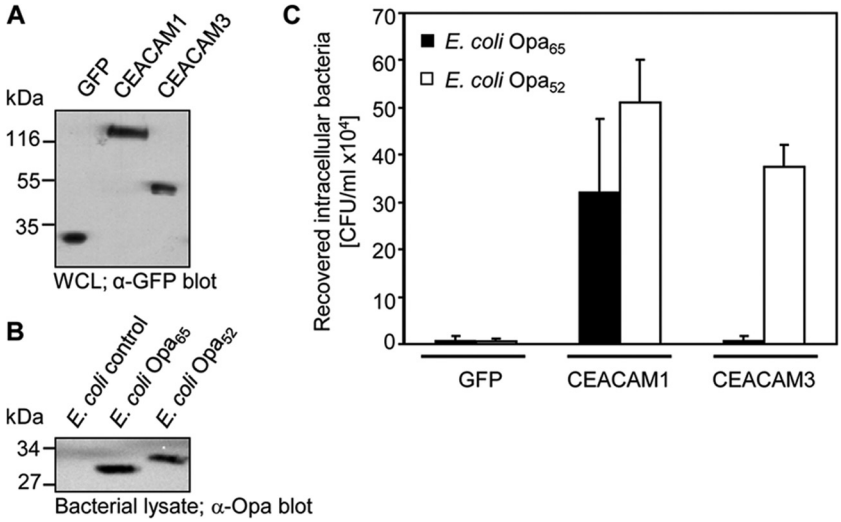

FIG 4 CEACAM1, but not CEACAM3, mediates internalization of *E. coli* VP1 Opa_{65} into human cells. (A) 293 cells were transfected with constructs encoding the indicated CEACAMs fused to GFP or GFP alone. After 48 h, cells were lysed and CEACAM expression was determined by Western blotting with a monoclonal anti-GFP antibody. WCL, whole-cell lysate. (B) Expression of MS11 and VP1 Opa proteins in *E. coli* was verified by Western blotting with a monoclonal anti-Opa antibody. (C) 293 cells were transfected as described for panel A, and 2 days later, cells were infected for 1 h with *E. coli* Opa_{52} or *E. coli* Opa_{65}, as indicated (MOI, 100). The number of viable intracellular bacteria was determined by gentamicin protection assays. Bars represent mean values ± SEMs of 3 independent experiments done in triplicate.

were visually selected. If several distinct opacity types could be distinguished, more than one Opa variant was picked for each strain and Opa protein expression was verified by Western blotting (Fig. 7A). Opa expression was comparable to expression of Opa_{52} in *N. gonorrhoeae* strain MS11, with the Opa2 variant of DGI strain 241 showing the highest Opa protein expression (~3-fold compared to the level of MS11 Opa_{52} expression). The soluble extracellular domains of CEACAM1 and CEACAM3 were produced (Fig. 7B) and employed to test CEACAM binding by the different gonococci. Interestingly, none of the six Opa variants from the four DGI strains bound to CEACAM3, whereas four Opa variants interacted with CEACAM1 (Fig. 7C). Furthermore, with the exception of strain 241 Opa2, no significant oxidative burst was detected for any of the Opa protein-expressing variants (Fig. 7D). Despite the strong expression of Opa protein in strain 241 Opa2, the total oxidative burst was still lower than that triggered by MS11 Opa_{52} (Fig. 7E). Together, our data suggest that a paucity of CEACAM3-binding Opa proteins might allow DGI-causing strains to escape innate detection and destruction by human granulocytes and might contribute to their ability to disseminate in the human body.

DISCUSSION

A minority of gonococcal strains has the ability to cause disseminating forms of disease, often accompanied by severe symptoms. Several phenotypic characteristics, including complement resistance, Opa protein-independent host cell invasion, and the AHU auxotrophy phenotype, have been related to the disseminating potential of these bacteria. Here, we describe an additional molecular trait which might contribute to the ability of *N. gonorrhoeae* strains to disseminate. Upon cloning and functional analyses of the Opa proteins of the DGI strain VP1, we could observe that this strain is able to engage CEACAMs present on host epithelial cells but lacks Opa proteins mediating recognition by the human granulocyte receptor CEACAM3. This is in sharp contrast to Opa proteins derived from *N. gonorrhoeae* strain MS11, which causes local infections and whose genome encodes Opa proteins with CEACAM3-binding potential. Our results with additional DGI isolates suggest that disseminating gonococcal strains escape the innate immune surveillance afforded by CEACAM3 recognition and that such strains might not be appropriately controlled in an opsonin-independent manner by human granulocytes.

CEACAM3 engagement by bacteria results in rapid phagocytosis and destruction of microbes by human granulocytes (13). Upon bacterial binding to CEACAM3, the immunoreceptor tyrosine-based activation motif (ITAM)-like sequence within the cytoplasmic domain of CEACAM3 becomes tyrosine phosphorylated by Src family kinases (39, 43). In turn, the phosphorylated tyrosine residue pY230 of CEACAM3 can serve as a docking site for the Rac guanine nucleotide exchange factor (GEF) Vav, which connects CEACAM3 engagement with pronounced GTP loading of the small GTPase Rac (13, 44, 45). Furthermore, tyrosine-phosphorylated CEACAM3 associates with the adaptor molecule Nck, which couples the WAVE complex via Nck-associated protein 1 (Nap1) to the clustered receptor (46). There, the CEACAM3-localized WAVE complex can be fully activated by Rac-GTP, triggering local actin polymerization and bacterial engulfment (12). Furthermore, the phosphorylated ITAM-like sequence of CEACAM3 recruits the regulatory subunit of phosphatidylinositol 3′-kinase (PI3K), which, together with the protein tyrosine kinase Syk, orchestrates the assembly and activity of the NADPH oxidase complex downstream of CEACAM3 clustering (34, 42). Together, CEACAM3-initiated phagocytosis and reactive oxygen production promote the opsonin-independent elimination and destruction of CEACAM3-binding bacteria. Therefore, avoiding CEACAM3-mediated recognition appears to be an effective means to escape this form of innate surveillance, and it is plausible that the lack of CEACAM3-binding Opa_{CEA} proteins in strain VP1

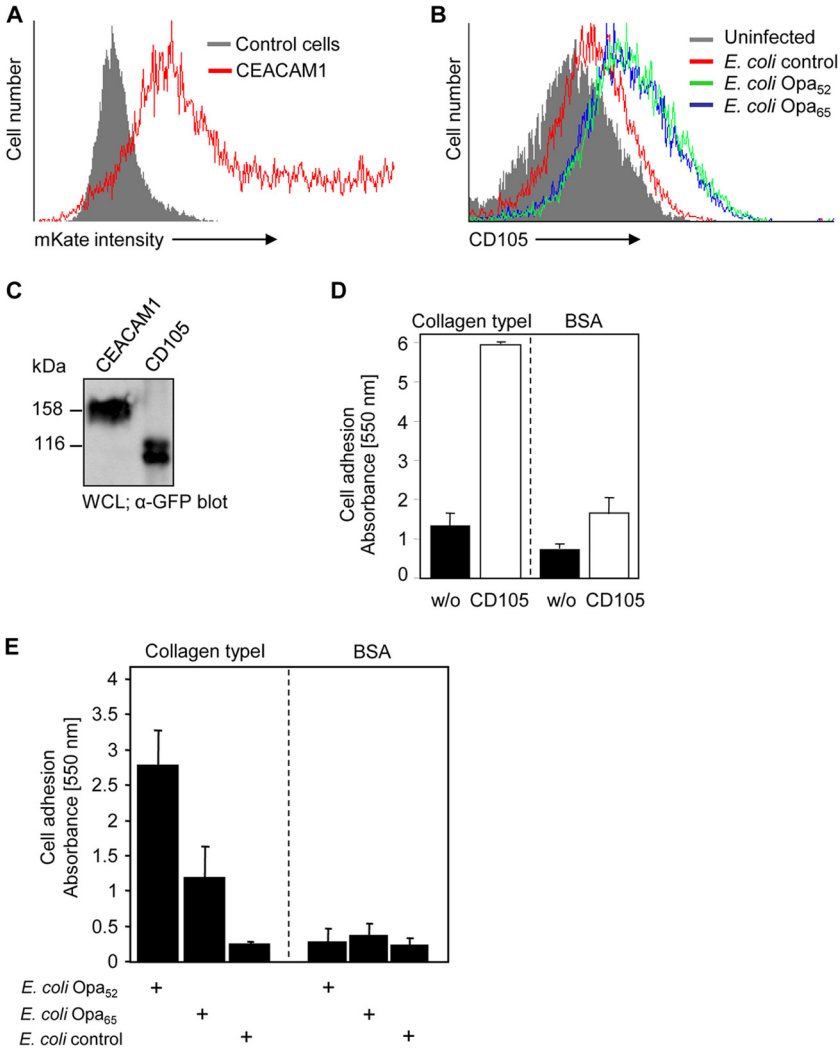

FIG 5 CEACAM1 stimulation leads to CD105 expression and increased cell adhesion. (A) 293 cells were transiently transfected with CEACAM1 fused to mKate and analyzed by flow cytometry for CEACAM1 expression. (B) Cells transfected as described for panel A were either left uninfected or infected for 6 h with the indicated *E. coli* strains. Infected cells were stained with a monoclonal antibody against CD105 and a secondary antibody conjugated to Alexa 488. CD105 expression was determined by flow cytometry, and 10,000 events were determined for each sample. Shown is a representative experiment repeated 3 times with similar results. (C) 293 cells were transfected with CEACAM1-GFP or CD105-GFP. Two days later, cells were lysed and analyzed by Western blotting with a monoclonal antibody directed against GFP. (D) Cells transfected as described for panel C were analyzed in assays for adhesion to either collagen or BSA. Adherent cells were fixed and stained with crystal violet, and staining intensity was determined after dye elution at 550 nm. Bars represent mean values ± SDs of 6 wells. (E) Cells transfected as described for panel C were infected for 12 h with the following strains: *E. coli* MS11 Opa_{52}, *E. coli* VP1 Opa_{65}, or *E. coli* lacking Opa protein expression (*E. coli* control). Cells were then employed in a cell adhesion assay as described for panel D. Bars represent mean values ± SDs of 6 wells.

might contribute to the ability of this gonococcal strain to disseminate.

Besides CEACAM3, two other pathogen-binding CEACAMs, CEACAM1 and CEACAM6, are present on human granulocytes. Despite the large amounts of CEACAM1 and CEACAM6, CEACAM3 is the major receptor responsible for opsonin-independent phagocytosis of CEACAM-binding bacteria by human granulocytes (34, 39, 41). For instance, phagocytosis of Opa_{CEA} protein-expressing gonococci by human granulocytes is strongly affected by pharmacological inhibitors of Src-family kinases, and such inhibitors diminish CEACAM3-mediated but not CEACAM6-mediated uptake in transfected cell lines (39, 40). Similarly, the glycosylphosphatidylinositol-anchored CEACAM6 and clustered CEACAM1 are located in cholesterol- and sphingolipid-rich membrane microdomains (lipid rafts), whereas CEACAM3 resides in the nonraft fraction of the membrane (33, 39). Accordingly, CEACAM1- and CEACAM6-mediated uptake of bacteria is sensitive to cholesterol-chelating agents such as methyl-β-cyclodextrin or nystatin, whereas CEACAM3-mediated phagocytosis is not compromised by cholesterol depletion (39). Most importantly, methyl-β-cyclodextrin treatment does not affect the opsonin-independent bacterial phagocytosis of Opa_{CEA} protein-expressing gonococci by primary human granulocytes (39). Experiments with murine myeloid cell lines stably expressing one or several human CEACAMs have also lent support to the idea that CEACAM3 is the critical receptor to orchestrate phagocyte responses (41). Clearly, in some situations, such as with very strong expression of a CEACAM1-binding Opa protein (as seen in

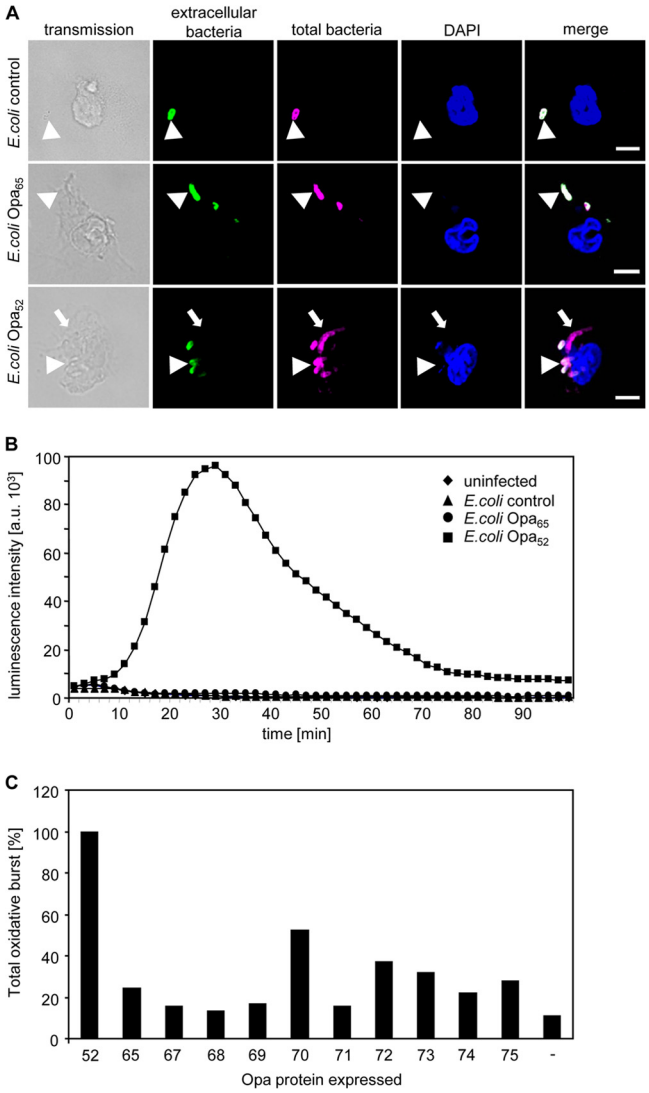

FIG 6 *E. coli* expressing MS11 Opa_{52} protein, but not VP1 Opa_{65} protein, is taken up by granulocytes and triggers an oxidative burst. (A) Primary human granulocytes were infected with *E. coli* lacking Opa protein expression (*E. coli* control), *E. coli* VP1 Opa_{65}, or *E. coli* MS11 Opa_{52} at an MOI of 40 for 30 min and fixed. Extracellular bacteria (green) were stained using polyclonal anti-*E. coli* LPS antibody in combination with a Cy2-conjugated secondary antibody. After cell permeabilization, bacteria were again stained using polyclonal anti-*E. coli* LPS antibody in combination with a Cy5-conjugated secondary antibody (pink) and the granulocyte nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Extracellular bacteria are marked by simultaneous Cy2 and Cy5 staining (arrowheads; green and pink), whereas intracellular bacteria are only stained by Cy5 (small arrow; pink). Bars, 5 μm. (B) Primary human granulocytes were isolated and infected at an MOI of 50 with *E. coli* control, *E. coli* MS11 Opa_{52}, or *E. coli* VP1 Opa_{65} or were left uninfected, and the oxidative burst was measured for 100 min. Shown is a representative experiment repeated 3 times with similar results. a.u., absorbance units. (C) Primary human granulocytes were infected at an MOI of 50 with *E. coli* expressing MS11 Opa_{52}, *E. coli* expressing distinct VP1 Opa proteins (Opa_{65} and Opa_{67} to Opa_{75}), or an *E. coli* control (−), and the oxidative burst was measured. To estimate the total amount of reactive oxygen produced, the area under the curve was determined. The graph shows a representative experiment. Similar results were obtained with granulocytes from three different donors.

this study for Opa2 of strain 241) or at a high density of CEACAM1-binding bacteria (41), it might be possible to trigger a phagocyte response by engaging CEACAM1. However, in most cases, CEACAM1 engagement by bacteria does not activate human granulocytes, underscoring the prominent role of CEACAM3 as the main mediator of CEACAM-triggered bactericidal processes.

Given the functional consequences of CEACAM3 recognition and the enormous variety of Opa protein alleles, it would be advantageous to be able to predict the CEACAM3-binding potential on the basis of the amino acid sequence of a given Opa protein. However, a consensus motif characteristic for CEACAM-binding Opa proteins has not been delineated yet. Earlier studies have suggested that receptor binding is determined by the variable portions of Opa proteins, in particular, the HV-1 and the HV-2 regions (10, 47). Opa protein variants with deletions in extracellular loops and chimeric Opa proteins have underscored the critical role of both HV regions (48). Intriguingly, the fusion of HV-1 and HV-2 region sequences derived from distinct CEACAM-binding Opa proteins often leads to the loss of CEACAM binding by the resulting chimeric protein (48, 49). This unexpected result indicates that particular sequences in HV regions could be necessary, but not sufficient, for CEACAM binding and rather suggests that both HV regions have to combine properly to form a functional CEACAM ligand (48, 49). With the new sequence and binding information of *N. gonorrhoeae* VP1 Opa proteins obtained in our current study, we have also tried to decipher a common sequence motif for CEACAM binding. However, looking at patterns of charged or hydrophobic amino acid residues in the HV-1 and HV-2 regions, we could not come up with a clear distinction between CEACAM-binding and nonbinding Opa proteins. In contrast, a short amino acid motif found in the HV-2 loop of several meningococcal Opa proteins has been shown to be critical for CEACAM binding in this protein context (49). Unfortunately, the identified GxI/V/LxQ motif is not present in the HV regions of CEACAM-binding gonococcal Opa proteins, including the CEACAM-binding Opa proteins of strain VP1 analyzed in the present study, limiting the predictive power of this amino acid motif.

The crystal structure of the human CEACAM1 N domain and the mapping of amino acid residues critical for Opa protein binding have revealed that the nonglycosylated side of the receptor, in particular, the C, C′, and C″ β strands, provides the binding interface on CEACAM1 (50, 51). Given the lack of a clear-cut consensus motif in CEACAM-binding Opa proteins from pathogenic *Neisseria* species, critical Opa protein residues involved in CEACAM binding might be revealed only by a cocrystal structure of ligand and receptor.

The difficulties in delineating a common CEACAM-binding motif in neisserial Opa proteins are in contrast to the delineation of the motif in other CEACAM-binding adhesins. Especially in the case of UspA1, the CEACAM-binding adhesin of the human pathogen *Moraxella catarrhalis*, the CEACAM-binding motif has been mapped to a linear peptide sequence in the UspA1 stalk (52, 53). As UspA1 is a member of the oligomeric coiled-coil adhesin (Oca) family (54) and completely unrelated to Opa proteins with regard to its tertiary structure, it is not possible to extrapolate from these findings. Therefore, it is still mandatory to characterize each Opa protein with respect to its CEACAM-binding properties. Major factors which limit the scalability of the functional analysis of

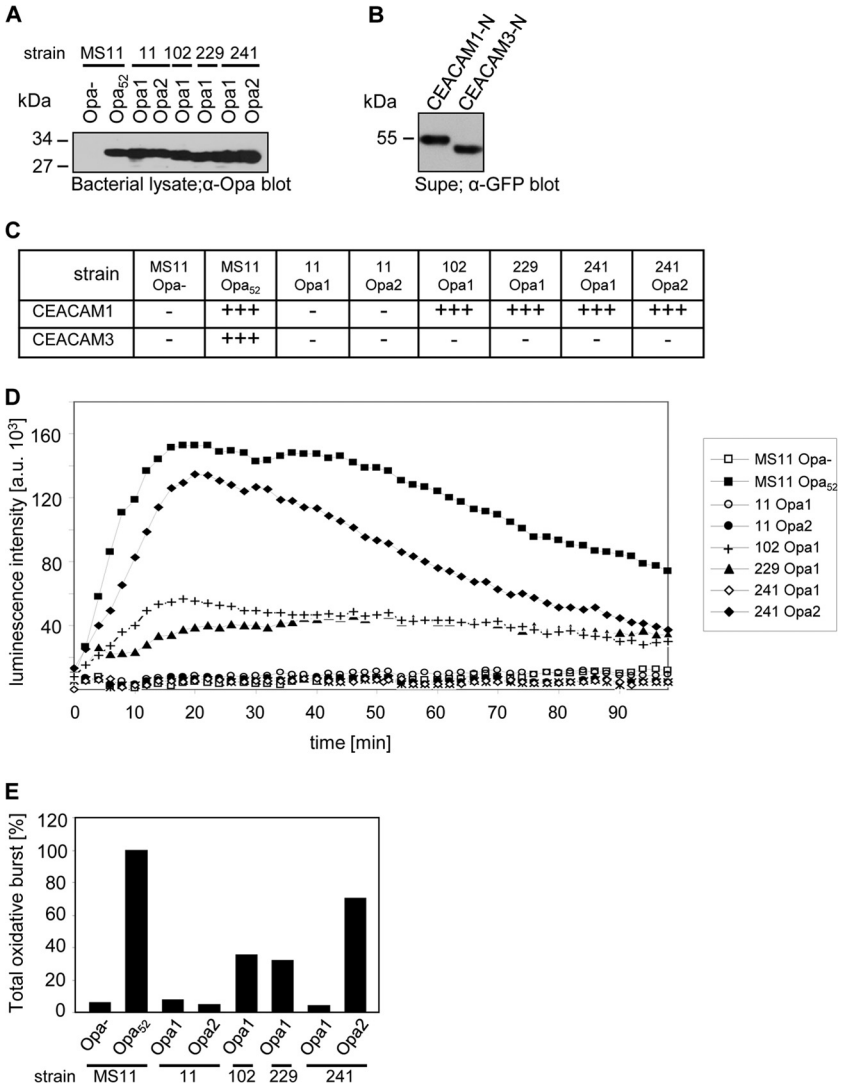

FIG 7 Most Opa protein variants from multiple DGI isolates interact with CEACAM1 but fail to bind the granulocyte receptor CEACAM3. (A) Opa protein expression of gonococcal strains isolated from DGI patients (strains 11, 102, 229, and 241) and gonococci expressing MS11 Opa_{52} or lacking Opa protein expression (Opa−) was verified by Western blotting with a monoclonal anti-Opa antibody. (B) The amount of GFP fusion protein of the amino-terminal CEACAM1 or CEACAM3 ectodomain was determined by Western blotting of culture supernatants with a monoclonal anti-GFP antibody. (C) Overview of the CEACAM-binding properties of the distinct Opa variants of the indicated gonococcal strains determined by pulldown assays with soluble GFP-tagged amino-terminal CEACAM domains. (D) Primary human granulocytes were isolated and infected at an MOI of 50 with the indicated Opa variants of strain MS11 or the DGI isolates, and the oxidative burst was measured for 100 min. Shown are the results of a representative experiment repeated 3 times with similar results. (E) To estimate the total amount of reactive oxygen produced, the areas under the curves of the experiment whose results are shown in panel D were determined. The total oxidative burst induced by MS11 Opa_{52}-expressing bacteria was set to 100%.

Opa protein binding specificities are the phase variation of Opa protein expression and the fact that a single strain contains multiple Opa genes. Therefore, it is necessary either to visually isolate and maintain single Opa protein-expressing strains or to clone and express a defined Opa protein.

To date, the cloning of the full repertoire of Opa proteins has been achieved for only a few neisserial strains. It is interesting to note that, similar to our results with the disseminating gonococcal strain VP1, a lack of CEACAM3-binding Opa proteins has been noticed for two meningococcal isolates (55). As these serogroup A and serogroup C strains of *N. meningitidis* were isolated from meningitis patients (56), they can also be regarded as disseminating bacteria. Together with the insight gained from the detailed functional analysis of Opa proteins derived from *N. gonorrhoeae*, a general pattern emerges: the genomes of strains that have disseminating potential (e.g., *N. gonorrhoeae* VP1) encode several Opa proteins with the ability to engage human CEACAMs present on epithelial cells, such as CEACAM1 and CEA. Therefore, such adhesins allow the bacteria to exploit CEACAMs for mucosal colonization, e.g., by enhancing the extracellular matrix binding capability of the infected epithelial cells (24, 25). On the contrary, these types of Opa proteins lack the ability to engage the closely related CEACAM3. Accordingly, these Opa proteins fail to trigger robust, opsonin-independent responses by human granulocytes. We refer to this group of Opa proteins as $Opa_{CEA\text{-}e}$ (where "e" represents "epithelial") to indicate that these adhesins specifically address

epithelial cells, and it appears that Opa proteins from other disseminating neisserial strains also fall in this category.

On the other hand, strains that are associated with localized infections have the ability to express one or more Opa proteins which engage not only CEACAMs on epithelial cells but also the granulocyte-restricted CEACAM3. Therefore, these types of adhesins are referred to as $Opa_{CEA\text{-}a}$ (where "a" stands for "all"), as they promote both mucosal colonization via interaction with CEACAMs on epithelial cells and strong stimulation of innate responses by granulocytes via CEACAM3.

Though on first sight it appears to be detrimental for the genomes of individual bacteria to encode and to express $Opa_{CEA\text{-}a}$ proteins, one has to consider the overall consequences of disseminating forms of disease for the host and for the pathogen population. In the case of *N. gonorrhoeae*, where the main route of transmission is via sexual intercourse, it is obvious that a patient with disseminated, severe forms of gonococcal infection is less likely to spread the microbe and to infect other hosts than a person with localized forms of disease or a person that lacks any symptoms at all. The same is true for meningitis patients, where systemic disease often has a fatal outcome, clearly setting a limit for such bacteria to spread to new hosts. Thinking in evolutionary terms, a disseminated form of the disease, probably supported by the exclusive expression of $Opa_{CEA\text{-}e}$ proteins, as seen for *N. gonorrhoeae* VP1 or as seen for the meningococcal strains analyzed, should be a dead end for these exquisitely adapted pathogens. Indeed, taking into account the large number of people colonized by meningococci or infected by gonococci, disseminated forms of disease are the exception (3, 57, 58). Therefore, one could speculate that a balance in the expression of $Opa_{CEA\text{-}a}$ and $Opa_{CEA\text{-}e}$ proteins might favor localized infection and reduce the chance of disseminated disease. As a result of the receptor and tissue tropism afforded by the functionally distinct $Opa_{CEA\text{-}a}$ and $Opa_{CEA\text{-}e}$ proteins, in the end, both the host and the pathogen might benefit. We propose that the functional analysis of Opa protein-receptor interaction combined with the epidemiological history of neisserial strains might provide important clues to understand the emergence of clonal neisserial lineages causing disseminated forms of disease.

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