

# Disease and Carrier Isolates of *Neisseria meningitidis* Cause G<sub>1</sub> Cell Cycle Arrest in Human Epithelial Cells

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Microbial pathogens have developed several mechanisms to modulate and interfere with host cell cycle progression. In this study, we analyzed the effect of the human pathogen *Neisseria meningitidis* on the cell cycle of epithelial cells. Two pathogenic isolates, as well as two carrier isolates, were tested for their ability to adhere to and invade into the epithelial cell lines Detroit 562 and NP69 and to modulate the cell cycle. We found that all isolates adhered equally well to both Detroit 562 and NP69 cells, whereas the carrier isolates were significantly less invasive. Using propidium iodide staining and 5-ethynyl-2'-deoxyuridine pulse-labeling, we provide evidence that meningococcal infection arrested cells in the G<sub>1</sub> phase of the cell cycle at 24 h postinfection. In parallel, a significant decrease of cells in the S phase was observed. Interestingly, G<sub>1</sub>-phase arrest was only induced after infection with live bacteria but not with heat-killed bacteria. By Western blotting we demonstrate that bacterial infection resulted in a decreased protein level of the cell cycle regulator cyclin D1, whereas cyclin E expression levels were increased. Furthermore, *N. meningitidis* infection induced an accumulation of the cyclin-dependent kinase inhibitor (CKI) p21<sup>WAF1/CIP1</sup> that was accompanied by a redistribution of this CKI to the cell nucleus, as shown by immunofluorescence analysis. Moreover, the p27<sup>CIP1</sup> CKI was redistributed and showed punctate foci in infected cells. In summary, we present data that *N. meningitidis* can interfere with the processes of host cell cycle regulation.

Recent studies have shown that many bacteria produce and secrete compounds, e.g., toxins and effectors, that interfere with the host cell cycle. These factors are summarized as “cyclomodulins” and have been proposed to be a new class of virulence-associated factors (1, 2). The cell cycle is a series of events that describe the growth, DNA replication, distribution of the duplicated chromosomes to daughter cells and division of a cell. It is divided into four phases: M phase (mitosis), G<sub>1</sub> (the period between mitosis and the initiation of nuclear DNA replication), S (the period of nuclear DNA replication), and G<sub>2</sub> (the period between the completion of nuclear DNA replication and mitosis). Cells in G<sub>1</sub> phase can enter a resting state called G<sub>0</sub>, which represents nongrowing and nonproliferating cells.

The progression from one cell cycle phase to another occurs in an orderly fashion and is regulated by different cellular proteins: key regulatory proteins are the cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, that are activated at specific points of the cell cycle (3). CDKs form complexes with different cyclins that are required at different phases of the cell cycle. Three D type cyclins—cyclin D1, cyclin D2, and cyclin D3—bind to CDK4 and to CDK6. CDK-cyclin D complexes are essential for entry in G<sub>1</sub> (4). Another G<sub>1</sub> cyclin is cyclin E, which associates with CDK2 to regulate progression from G<sub>1</sub> into S phase (5). Downstream targets of CDK-cyclin complexes include the retinoblastoma protein (pRB) and E2F transcription factors. CDK activity can be counteracted by cell cycle inhibitory proteins, called CDK inhibitors (CKI), which bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity. CKIs are classified into two groups, the INK4 and Cip/Kip families. INK4 family members bind only to CDK4/6 and inhibit their activities, whereas Cip/Kip family members (including p21<sup>WAF1/CIP1</sup>, p27<sup>CIP1</sup>, and p57<sup>CIP2</sup>) can inhibit the activities of G<sub>1</sub> CDK-cyclin complexes and, to a lesser extent, the CDK1-cyclin B complex (6, 7).

During coevolution with their hosts, bacteria have established

multiple mechanisms that allow them to interfere with cell proliferation. During the last decade, a growing family of bacterial effectors and toxins has been described that interferes with the host cell cycle (1, 2, 8, 9). The cytolethal distending toxin of *Escherichia coli* was the first bacterial toxin described to act as a “cyclomodulin” and has been shown to cause growth arrest at the G<sub>2</sub>/M phase (10). Further candidates are the cycle inhibiting factors (Cifs) produced by enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC), that trigger an irreversible cell cycle arrest at G<sub>2</sub> with complete inhibition of mitosis by inhibition of the CDK1-cyclin B complex, whose activation is necessary for the cell cycle G<sub>2</sub>/M transition (11). Other than G<sub>2</sub> arrest, Cif also induces G<sub>1</sub> cell cycle arrest in a process that involves the stabilization of the CKIs p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup> (12). Whereas these bacterial “cyclomodulins” induce cell cycle arrest, other bacterial toxins can also induce DNA replication and cell proliferation (1). These include the *Pasteurella multocida* toxin PMT (13), which upregulates cyclins D and E and p21<sup>WAF1/CIP1</sup>; the cytotoxic necrotizing factors from *E. coli* (14); the dermonecrotic toxin from *Bartonella* spp. (14); and CagA from *Helicobacter pylori* (15). Finally, alteration of

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TABLE 1 *N. meningitidis* strains used in this study<sup>a</sup>

Strain	Serogroup	ST	cc	Epidemiology	Source	Country	Yr	Reference	Genome accession no.
MC58	B	74	32	Invasive	IMD	United Kingdom	1983	73	AE002098
8013/clone12	C	177	18	Invasive	IMD	France	1989	74	FM999788
α4	B	19	18	Carriage	Carrier	Germany	1999	19	
α711	B	32	32	Carriage	Carrier	Germany	2000	19	

<sup>a</sup> ST, sequence type; cc, clonal complex; IMD, invasive meningococcal disease.

cell cycle progression has also been observed during pathogen-plant interaction (16).

*Neisseria meningitidis*, the meningococcus, is a Gram-negative organism and an obligate human pathogen. Meningococci are often found in the nasopharynx as asymptomatic colonizers and carriage rates vary between 10 and 35% for healthy adults (17–19). In populations with individuals in close contact, such as university students or military recruits, carriage rates of ca. 70% have been found (20). For colonization of the human nasopharynx, the microorganism must adhere to the mucosal surface, utilize locally available nutrients, and evade the human immune system. *N. meningitidis* expresses a range of structures and molecules that facilitate adhesion and invasion, including the type IV pili, the outer membrane proteins Opa and Opc, and a number of newly identified minor adhesion or adhesion-like proteins (21–28). Compared to the carriage rate, meningococcal disease is a rare event, and disease rates vary in different geographic regions of the world. The mechanisms that drive the colonization state of the organism into a disease state are still not entirely clear. It seems that a combination of bacterial virulence factors and host susceptibility, including age, smoking, prior viral infection, and genetic polymorphisms (29, 30), may facilitate meningococcal disease.

We have recently shown that *N. meningitidis* can interfere with the cell cycle of brain endothelial cells (31), inducing an accumulation of infected cells in the S phase, which is triggered by the major adhesins/invasins of *N. meningitidis*, the Opa proteins and the Opc protein (31). Mechanistically, cell cycle arrest could be correlated to an increased expression of the CKI p21<sup>WAF1/CIP1</sup> (31). In the present study, we analyzed the effect of *N. meningitidis* on the cell cycle of epithelial cells. We show that infection with *N. meningitidis* caused a G<sub>1</sub> cell cycle arrest, which correlated with an accumulation of CKI p21<sup>WAF1/CIP1</sup> protein levels and a redistribution of this CKI into the nucleus. Furthermore, meningococcal infection resulted in a decreased cyclin D1 and increased cyclin E protein amount. In addition to our previous study, we extended our analyses and included two pathogenic and two genetically related carrier isolates to decipher whether disease and carrier isolates differ in their ability to interfere with the host cell cycle of epithelial cells.

## MATERIALS AND METHODS

**Bacterial strains, mutants, and culture conditions.** *Neisseria meningitidis* strain MC58, a serogroup B isolate (United Kingdom, 1983) of the sequence type 74 (ST-74) (ST-32 clonal complex [cc]) was kindly provided by E. R. Moxon. The nonencapsulated mutant MC58 *siaD* has previously been described (32). *N. meningitidis* serogroup C strain 8013/clone12 (ST-18cc, ST-177) was kindly provided by M. Taha (33). *N. meningitidis* strain α4 (ST-18cc, ST-19) and strain α711 (ST-32cc, ST-32) were isolated during the Bavarian meningococcal carriage study (19) (the strains and mutants are summarized in Table 1).

All meningococcal isolates were routinely grown on commercially acquired Columbia blood agar plates (bioMérieux) at 37°C in a humidified

5% CO<sub>2</sub> incubator and were cultured in proteose-peptone medium (PPM) supplemented with 1× Kellogg's supplements I and II, 0.01 M MgCl<sub>2</sub>, and 0.005 M NaHCO<sub>3</sub> (PPM+). Meningococcal supernatants were prepared as follows: Dulbecco modified Eagle medium (DMEM) with 1× Kellogg's supplements I and II was inoculated with meningococcal strain MC58, 8013/12, α4, or α711, followed by incubation for 2 h at 37°C at 200 rpm. The optical density at 600 nm (OD<sub>600</sub>) was measured, and a second culture was inoculated at an OD<sub>600</sub> of 0.05, followed by incubation overnight at 37°C with 200-rpm shaking. The following day, the culture was centrifuged at 4,000 × g for 20 min at 4°C, and the supernatant was sterilized through a 0.2-μm-pore size filter. The supernatants were transferred to a 20-ml VivaSpin column (molecular weight cutoff, 10,000; Sartorius) and centrifuged at 4,000 × g for 20 min at 20°C. Concentrated proteins were removed from the concentrator pocket and analyzed by SDS-PAGE as described below. The supernatants were stored at –20°C until use.

**Epithelial cell lines.** The cell line Detroit 562 (human pharyngeal cells; ATCC CCL-138) was maintained in DMEM with GlutaMAX (Life Technologies) supplemented with 10% fetal calf serum (FCS; Life Technologies), 4 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), and 1× nonessential amino acids (GE Healthcare Europe GmbH, Freiburg, Germany) at 37°C and 5% CO<sub>2</sub>. Nasopharyngeal epithelial cell line NP69 (NP69 cells harboring *SV40T*) was kindly provided by George Sai Wah Tsao (University of Hong Kong) (34). Keratin profiles and studies of the response to TGFβ1 treatment have shown that NP69 cells retain the characteristics of primary nasopharyngeal cells. Cells were maintained in serum-free keratinocyte medium (KSFM), supplemented with human recombinant epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 μg/ml) (Life Technologies). The cells were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Reagents and antibodies.** The following antibodies and reagents were used in this study: anti-β-actin (catalog no. 4967) and anti-p21<sup>WAF1/CIP1</sup> (clone 12D1; catalog no. 2947) (both from Cell Signaling); anti-cyclin E (clone HE12; sc-247; Santa Cruz Biotechnologies) and anti-cyclin D1 (clone EP272Y; 04-221; Merck) for Western blotting; and anti-p21<sup>WAF1/CIP1</sup> (clone 12D1; 2947; Cell Signaling) and anti-p27<sup>CIP1</sup> (clone G173-534 [RUO]; 554069; BD Biosciences) for immunofluorescence analyses. Cy3-conjugated rabbit anti-mouse (315-166-047) was from Dianova, Alexa 488-conjugated goat anti-rabbit (A-11008) and DAPI (4',6'-diamidino-2-phenylindole; D1306) were purchased from Life Technologies, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG/IgM (115-035-044) and HRP-goat anti-rabbit IgG (111-035-006) were from Dianova.

**Gentamicin protection assay.** Gentamicin protection assays were conducted as described previously (32). Briefly, 5 × 10<sup>4</sup> Detroit 562 cells and 1 × 10<sup>5</sup> NP69 cells per well were seeded in 24-well plates. Cells were infected with bacteria at a multiplicity of infection (MOI) of 30 for the indicated time points. Infections were carried out in DMEM–10% FCS for Detroit 562 cells and supplemented KSFM for NP69 cells. The medium was then replaced with DMEM or supplemented KSFM containing 200 μg/ml gentamicin. After 2 h of incubation in gentamicin-containing medium, the cells were lysed by the addition of 1% saponin in DMEM or supplemented KSFM for 15 min. Suitable dilutions were plated in duplicate on blood agar plates to determine the numbers of recovered viable bacteria.

**Real-time PCR.** Total RNA was extracted using the RNeasy minikit (Qiagen), including an on-column DNase digestion step using an RNase-free DNase set (Qiagen). The quality of extracted RNA was assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies) using an RNA Nano-Chip for eukaryotic RNA according to the manufacturer's protocol. Only RNA samples with an RNA integrity number that was  $\geq 9$  were used for further analysis (35). First-strand cDNA was synthesized from 1.0  $\mu\text{g}$  of total RNA using SuperScript II reverse transcriptase (Thermo Scientific) and a QIAquick PCR purification kit (Qiagen). The following primers were used for RT-PCR: forward cyclin D (5'-CGG TAG TAG GAC AGG AGG TT-3') and reverse cyclin D (5'-CTG TGC CAC AGA TGT GAA GT-3'); forward cyclin E (5'-AGC GGT AAG AAG CAG AGC AG-3') and reverse cyclin E (5'-CGC TGC AAC AGA CAG AAG AG-3'); and forward human GAPDH (5'-GCA CCG TCA AGG CTG AGA AC-3') and reverse human GAPDH (5'-ATG GTG GTG AAG ACG CCA GT-3'). Transcribed cDNA and primers were mixed with RT<sup>2</sup> SYBR green ROX qPCR Mastermix (Qiagen) and RNase-free water (Qiagen), and 25  $\mu\text{l}$  was loaded into each well of a 96-well plate. Quantitative real-time PCR was performed on a StepOnePlus real-time system (Applied Biosystems). For PCR, the mixture was denatured at 95°C for 3 min, and the target genes were amplified by 35 cycles of reaction: cyclin D1 (95°C for 30 s, 61°C for 30 s, and 72°C for 1 min), cyclin E (95°C for 30 s, 61°C for 30 s, and 72°C for 1 min), or GAPDH (95°C for 30 s, 61°C for 30 s, and 72°C for 1 min). Three independent experiments were performed for infected and uninfected cells at each time point. The gene expression values were normalized against GAPDH and were calculated according to the comparative  $\Delta C_T$  method (36). We used a cutoff of  $\geq 1.5$ -fold change and a *P* value of  $\leq 0.01$ .

**Flow cytometry assay.** Cell cycle was monitored by propidium iodide (PI) staining, followed by flow cytometry as previously described (31). Briefly, Detroit 562 or NP69 cells were seeded in six-well plates at  $2 \times 10^5$  cells per well for 24 h and allowed to grow to 50 to 60% confluence, since tight confluence can induce a cell cycle arrest. Detroit 562 cells or NP69 cells were infected with bacteria in DMEM–10% FCS or supplemented KSM, respectively, at an MOI of 100, and the bacteria were allowed to adhere for 6 h at 37°C and 5% CO<sub>2</sub>. Subsequently, the cells were washed with 1  $\times$  PBS, and the medium was replaced with DMEM–10% FCS or supplemented KSM containing 200  $\mu\text{g}/\text{ml}$  gentamicin to prevent bacterial overgrowth. The same infection conditions were used for the EdU cell proliferation assay, the immunofluorescence studies, and the Western blot analyses. In some experiments, cells were exposed to heat-inactivated bacteria or meningococcal supernatants.

Treated cells were harvested by trypsinization, washed twice with 1 ml of 1  $\times$  PBS using centrifugation, and then fixed by adding 70% ethanol dropwise at 4°C. Next, the cells were incubated for at least 60 min at 4°C, centrifuged, and washed once with 1  $\times$  PBS, followed by lysis in 0.05% Triton X-100 (Roth), treatment with 100  $\mu\text{g}/\text{ml}$  RNase A (Roche Diagnostic) for 30 min at 37°C, and staining with PI (100  $\mu\text{g}/\text{ml}$ ; Sigma) for 30 min at 37°C, protected from light. The cells were subsequently analyzed on a FACSCalibur flow cytometer (BD Biosciences), and BD CellQuest Pro software (BD Biosciences) was used for data acquisition. To determine the proportion of cells at each stage of the cell cycle, the cell cycle curve-fitting option of the software package WEASEL v3.1 (Walter and Eliza Hall Institute of Medical Research) was used.

**EdU cell proliferation assay.** The quantification of 5-ethynyl-2'-deoxyuridine (EdU) incorporation into replicating DNA was performed using the Click-iT EdU flow cytometry assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions with the following modifications. A total of  $2 \times 10^5$  cells per well were plated in six-well plates and grown for 24 h at 37°C. The cells were infected with bacteria (MOI of 100) as described above. After 22 h of infection, the cells were labeled with 20  $\mu\text{M}$  EdU, followed by incubation for another 2 h. Afterward, the cells were fixed and permeabilized as described above, and click labeled with Alexa Fluor 488 azide (Thermo Fisher Scientific) for 30 min in the presence of copper(II) sulfate and a reducing agent to afford the

copper-catalyzed click reaction (37). In addition, the cells were stained with 1  $\mu\text{l}$  of FxCycle Far Red (Invitrogen) for 30 min at 4°C. EdU and FxCycle Far Red measurements were performed using a BD FACSCalibur flow cytometer. Alexa Fluor 488 signals were measured on FL1 with a 530/30-nm band-pass filter, and FxCycle Far Red on FL3 was measured with a 670-nm long-pass filter. Sample measurements were performed with BD CellQuest Pro software.

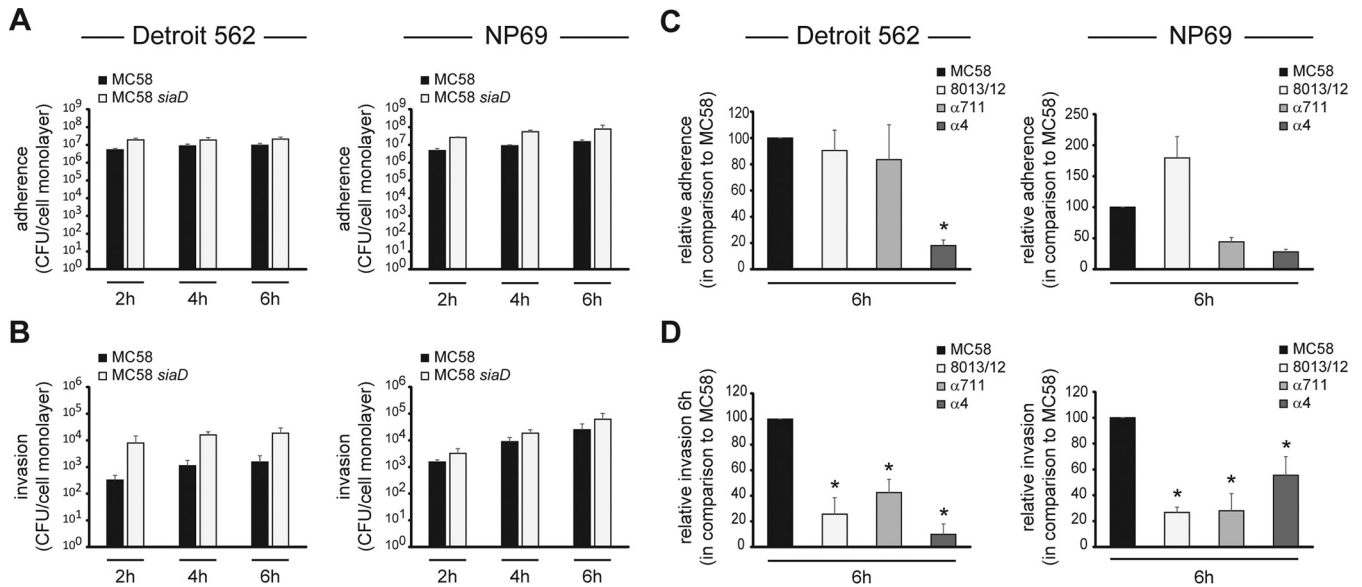
**Immunofluorescence assay.** The procedure for immunofluorescence was performed as described previously (31). Briefly, Detroit 562 cells were grown on glass coverslips, infected as described above, washed with 1  $\times$  PBS, and fixed with 3.7% formaldehyde for 20 min. After washing, the cells were permeabilized with 0.1% Triton X-100 (Roth) for 15 min and blocked with blocking buffer (BB; PBS, 1% FCS, and 2% bovine serum albumin) for at least 45 min at 4°C. Monoclonal anti-p21<sup>WAF1/CIP1</sup> IgG antibody was used at a 1:400 dilution in BB, and monoclonal anti-p27 IgG antibody was used at a 1:50 dilution in BB. A goat anti-rabbit Alexa Fluor 488-labeled secondary antibody and a rabbit anti-mouse Cy3-labeled secondary antibody were used at a 1:200 dilution. Nuclei were stained with DAPI at a 1:10,000 dilution in PBS, and the cells were examined by fluorescence microscopy using a Zeiss Axio Imager Z1 fluorescence microscope at a  $\times 40$  or a  $\times 63$  magnification. Images were processed with ImageJ software and documented using Adobe Photoshop CS.

**Western blot analysis.** The levels of cyclins and cell cycle regulating proteins were examined by using cell extracts prepared as described recently (31). All primary antibodies (p21<sup>WAF1/CIP1</sup>, cyclin D1, and cyclin E) were used at 1:1,000 in TBST (0.1% Tween). Western blots are shown for one of three independent experiments. Band analysis for each protein was quantified using ImageJ software. The area under the curve (AUC) of the specific signal was corrected for the AUC of the  $\beta$ -actin loading control before comparison for changes (recorded as the *n*-fold change) in a two-step normalization method. First, the relative densities were calculated by dividing the intensity of the protein band by the intensity of the corresponding  $\beta$ -actin band. Second, all of the relative band intensities were adjusted to the band intensity obtained from uninfected control cells. SDS-PAGE and Western blotting for pilin, Opa, and Opc expression was conducted as previously described (32) using the SM1 monoclonal antibody specifically recognizing class I pilin types (38), the 4B12/C11 pan-Opa antibody, and the Opc-specific monoclonal antibody B306 (39).

**Statistical analysis.** Statistical differences between groups were calculated using the Student unpaired *t* test (two tailed). *P* values of  $\leq 0.05$  were considered statistically significant.

## RESULTS

**Adherence to and invasion of the nasopharyngeal epithelial cell lines Detroit 562 and NP69 by invasive and carriage *N. meningitidis* isolates.** We have previously shown that *N. meningitidis* is able to interfere with the host cell cycle of human brain microvascular endothelial cells after infection (31). To investigate whether *N. meningitidis* infection might also result in an alteration of the cell cycle of nasopharyngeal cells, two cell lines were implemented, the pharyngeal carcinoma epithelial cell line Detroit 562 and NP69 cells. NP69 cells were established from primary nonmalignant nasopharyngeal epithelial cells and were transduced with the SV40T antigen (34). Keratin profiles and response to TGF $\beta$ 1 treatment have shown that NP69 cells retained characteristics of primary nasopharyngeal cells. First, adhesion and invasion properties of two disease and two carriage meningococcal isolates were quantified by using gentamicin protection assays as described previously (32, 40). Detroit 562 and NP69 cells were infected with the serogroup B disease strain MC58, a corresponding isogenic unencapsulated mutant strain MC58 *siaD*, the serogroup C disease isolate 8013/clone12, and the two serogroup B carrier isolates  $\alpha 711$  and  $\alpha 4$  (Table 1). *N. meningitidis* MC58 was chosen as a prototype strain belonging to the ST-32 clonal complex (cc),



**FIG 1** Carrier isolates and strain 8013/clone12 are less invasive than meningococcal disease isolates in epithelial host cells. Adhesion to and invasion of *N. meningitidis* into Detroit 562 and NP69 cells were assessed over a 6 h infection period. (A and B) Adherence to (A) and invasion of (B) both cell lines of serogroup B strain MC58 (■) and unencapsulated strain MC58 *siaD* (□) were determined by gentamicin protection assays at the time intervals indicated. Both cell lines were infected at an MOI of 30. (C and D) Adherence to (C) and invasion of (D) serogroup C disease isolate *N. meningitidis* 8013/clone12 and two serogroup B carrier isolates α711 and α4 (MOI 30) were determined by gentamicin protection assays at 6 h p.i. and are represented as relative data compared to MC58. The data show mean values ± the standard deviation (SD) of three independent experiments conducted in duplicate. \*, *P* < 0.05.

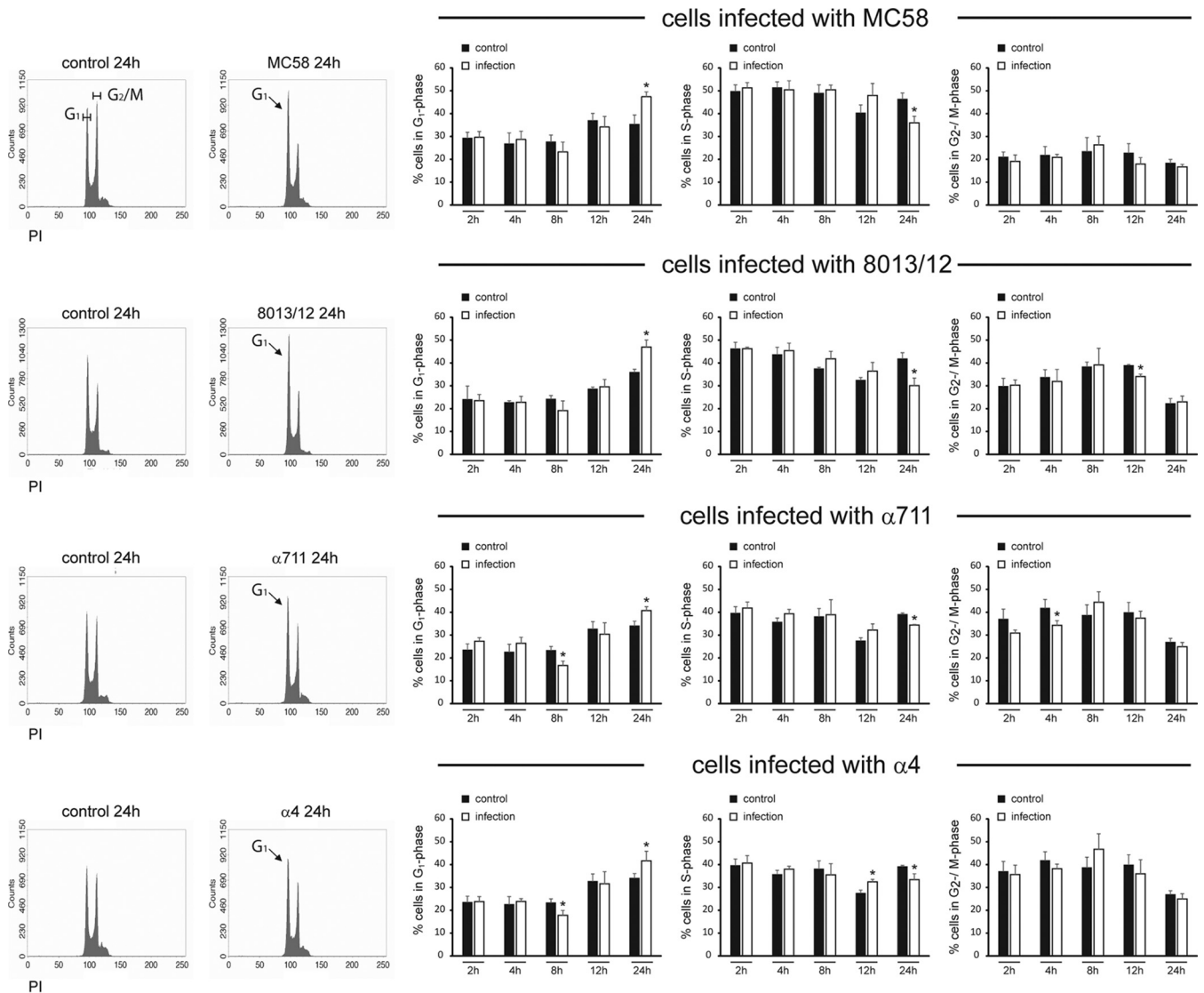
whereas strain α711 was chosen as a carrier isolate related to MC58 from the ST-32 cc (6). Furthermore, *N. meningitidis* isolate 8013/clone 12 (also known as clone 12 or 2C43) was included as a prototype ST-18 cc disease strain, while isolate α4 was chosen as a carrier isolate related to 8013/clone12. The invasion and adherence kinetics were estimated at 2, 4, and 6 h postinfection (p.i.), and the cells were infected at an MOI of 30 (Fig. 1).

Meningococci adhered very effectively to both Detroit 562 and NP69 cells, reaching a maximum of about 1 × 10<sup>7</sup> adherent bacteria per cell monolayer for strain MC58 at 6 h p.i. (Fig. 1A). In addition to adherence, the meningococcal wild-type strain MC58 elicited a time-dependent increase of bacterial uptake ranging from 3.2 × 10<sup>2</sup> at 2 h p.i. up to 1.54 × 10<sup>3</sup> at 6 h p.i. for Detroit 562 cells and from 1.53 × 10<sup>3</sup> at 2 h p.i. up to 2.5 × 10<sup>4</sup> at 6 h p.i. for NP69 cells. The unencapsulated strain MC58 *siaD* showed a significantly higher rate of invasion into Detroit 562 cells (about 7.9 × 10<sup>3</sup> at 2 h p.i. and 1.9 × 10<sup>4</sup> at 6 h p.i.) than its capsulated parent strain according to previously published data (Fig. 1) (32). Interestingly, the two carriage isolates and isolate 8013/clone12 were significantly less invasive than *N. meningitidis* MC58 into both Detroit 562 and NP69 cells. Invasion rates of about 25.6% invasion for 8013/clone12, 42.5% for α711%, and 9.8% for α4 were observed compared to MC58 at 6 h p.i. for Detroit 562 cells and 26.7% invasion for 8013/clone12, 28.0% for α711, and 55.5% for α4 using NP69 cells (Fig. 1D). The meningococcal isolates used in the present study were characterized for the expression of some of the major adhesins/invasins and phenotypes by Western blotting (see Fig. S1 in the supplemental material). This analysis showed that all four strains expressed class I pilin. (there was no change in the migration or reactivity of pilin from MC58, 8013/clone12, α711, and α4, respectively). However, 8013/clone12 did not express any Opa proteins, as analyzed by Western blotting and

8013/clone12, α711, and α4 did not react with the Opc-specific monoclonal antibody B306 either due to a deletion of the *opc* gene (8013/clone12 and α4 [data not shown]) or as a result of transcriptionally regulated phase variation (α711) (see Fig. S1B in the supplemental material), which is mediated by a variable polycytidine stretch in the promoter region of the gene (41).

***N. meningitidis* infection causes an accumulation of epithelial cells in the G<sub>1</sub> phase of the cell cycle.** To investigate the effects of pathogenic and carrier *N. meningitidis* isolates on the host cell cycle of human epithelial cells, asynchronous cultures of Detroit 562 and NP69 cells were either left uninfected or were infected with the disease isolates MC58 and 8013/clone12 or the two carrier isolates α711 and α4 using an MOI of 100 for 24 h (see Materials and Methods), and the DNA content of infected cells was determined by propidium iodide (PI) staining and subsequent flow cytometry analyses. The cells were infected over a maximum time period equal to the doubling time of the cell, which was estimated as 24 h for Detroit 562 cells and 30 h for NP69 cells. The cells were harvested at 2, 4, 8, 12, and 24 h p.i. or at 3, 6, 12, 24, and 30 h p.i., respectively, for PI staining.

Unfortunately, NP69 cells were not suited for PI staining when infected with *N. meningitidis* at time points later than 12 h p.i. in our hands. A significant fraction of colonies of clumped up cells was observed and resulted in an increase of super G<sub>2</sub> fraction, which interfered with our cell cycle curve-fitting analysis. We therefore focused in the following assays on Detroit 562 cells. As a consequence of meningococcal infection, Detroit 562 cells displayed a significant accumulation of cells in the G<sub>1</sub> phase at 24 h p.i. in response to the pathogenic isolates MC58 and 8013/clone12 to a level of about 47.4% for MC58 and 47.0% for 8013/clone12 compared to 35.4 and 35.9% in control cells (Fig. 2). Interestingly, carrier isolates also induced an accumulation of cells in the G<sub>1</sub>

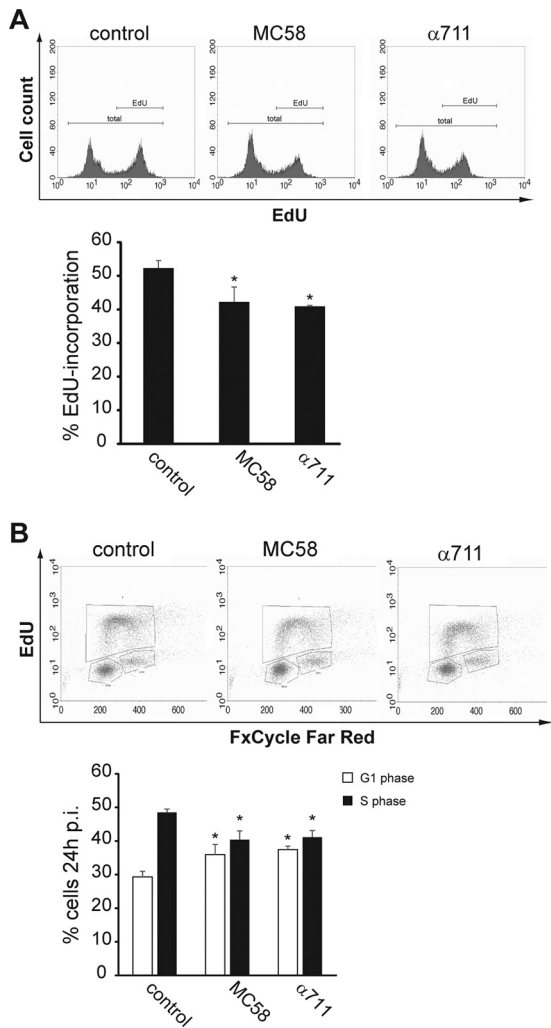


**FIG 2** Both pathogenic *N. meningitidis* isolates and carrier strains cause an accumulation of epithelial cells in the G<sub>1</sub> phase. Detroit cells either were left uninfected (control cells) or were infected with *N. meningitidis* strain MC58, 8013/clone12, α711, or α4 at an MOI of 100. Cells were collected at the time points indicated for propidium iodide (PI) staining and subsequent flow cytometry analysis of the cell cycle. In the left panel, representative histograms (24 h p.i.) for uninfected control cells and cells infected with the different meningococcal isolates are shown. The G<sub>1</sub> and G<sub>2</sub>/M phases are indicated. In the right panel, the histograms were analyzed with the software package WEASEL v3.1, and the percentages of the cells in the G<sub>1</sub>, S, and G<sub>2</sub>/M phases are shown. The results are presented as the means and the SD from three independent experiments. The statistical significance was determined for comparison between uninfected control cells and *N. meningitidis*-infected cells at each time point. \*, *P* < 0.05.

phase of about 40.7% for isolate α711 and 41.6% for isolate α4, respectively, compared to about 34.0% in control cells (Fig. 2). In parallel, a significant reduction of cells in the S phase was detected at 24 h p.i. (35.9% for MC58, 30.1% for 8013/clone12, 34.4% for α711, and 33.4% for α4, compared to about 41% of the cells in S phase for uninfected control cells) (Fig. 2). These results are indicative of an arrest at the G<sub>1</sub> phase of epithelial cells in response to both pathogenic and carrier isolates.

To verify that meningococcal infection arrested Detroit 562 cells in G<sub>1</sub> phase and reduced the number of cells in S phase, we next used MC58 and carrier isolate α711 and used an improved cell proliferation assay based upon the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) for the direct measurement of cells in the S phase. In this approach, EdU was added, incorporated

into replicating DNA and measured after treatment with the Alexa Fluor 488 Click-iT kit to label newly synthesized DNA during arrest. FxCycle Far Red was used to quantify nuclear DNA. In comparison to uninfected control cells, we found a significantly smaller amount of incorporated EdU in Detroit 562 at 24 h p.i. when cells were infected with MC58 (42.2%) or α711 (40.9%) compared to control cells (51.1%) (Fig. 3A), indicating that *N. meningitidis*-infected Detroit 562 cells remained mostly in the G<sub>1</sub> phase. Moreover, the percentages of cells in each stage of the cell cycle was determined by FxCycle Far Red staining, which revealed, that the reduction of EdU-positive cells during bacterial infection was due to the sequestration of cells in the G<sub>1</sub> phase. The cell cycle distribution pattern obtained by this technique was in line with the data obtained by PI staining and



**FIG 3** Meningococcal infection decreases the number of Detroit 562 cells in the S phase. Detroit 562 cells were either left uninfected or were infected with MC58 or α711 (MOI of 100) for a 24-h time period. Cell proliferation analysis was performed using a Click-iT EdU Alexa Fluor 488 flow cytometry assay kit and FxCycle Far Red stain. During the final 2 h of infection, Detroit 562 cells were treated with 20 μM EdU, harvested, and then analyzed by flow cytometry. EdU was stained with Alexa Fluor 488 azide according to the manufacturer's protocol, followed by staining with 1 μM FxCycle Far Red stain for DNA content analysis. The cells were then analyzed by flow cytometry using either 488-nm excitation for the EdU Alexa Fluor 488 dye or 633-nm excitation for the FxCycle Far Red stain. (A, upper panel) The results of a representative experiment are shown. The complete population (total) and the EdU-positive (EdU) populations are indicated. (Lower panel) The results of a statistical analyses of the percentages of the cell population incorporating EdU in uninfected control cells, MC58-infected cells, or α711-infected cells from three independent experiments are shown. The data are shown as means ± the standard deviations (\*, *P* < 0.05). (B, upper panel) The results of a representative experiment are shown, and cells in the G<sub>1</sub>, S, and G<sub>2</sub>/M phases are gated in boxes. (Lower panel) The results of a statistical analyses of the percentages of the cell population in the S phase in uninfected control cells, MC58-infected cells, or α711-infected cells from three independent experiments are shown. The data are shown as means ± the standard deviations (\*, *P* < 0.05).

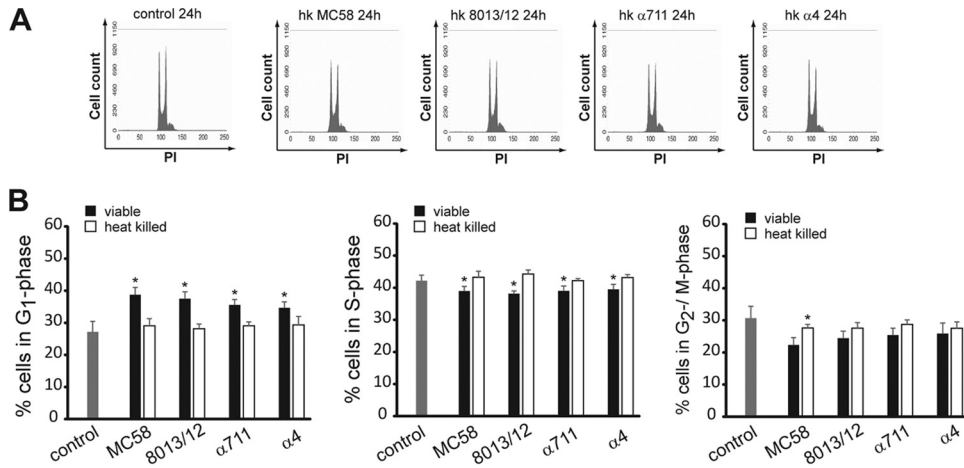
proved that meningococcal infection caused an arrest of host cells in the G<sub>1</sub> cell cycle phase (Fig. 3B).

**Live but not heat-killed meningococci induce an accumulation of epithelial cells in the G<sub>1</sub> phase of the cell cycle.** We next

examined whether Detroit 562 cells were also arrested in G<sub>1</sub> phase after treatment with heat-killed bacteria. Detroit 562 cells were exposed to both live and heat-killed meningococcal isolates MC58, 8013/clone12, α711, and α4 for 24 h and cellular DNA content was measured by PI staining as described above. Although live bacteria induced an accumulation of cells in the G<sub>1</sub> phase as demonstrated earlier (Fig. 4), heat-killed (hk) bacteria of all *N. meningitidis* isolates had no effect on the cell cycle distribution (29.1% for hk MC58, 28.2% for hk 8013/clone12, 29.1% for hk α711, and 29.3% for hk α4, compared to 27.2% of the cells in the G<sub>1</sub> phase for uninfected control cells) (Fig. 4). These findings suggested that the alteration of the cell cycle might be attributed to heat-labile bacterial proteins or might be due to less internalized bacteria that are able to initiate various signaling cascades.

**Meningococcal supernatants do not account for G<sub>1</sub>-phase arrest of the cell cycle in Detroit 562 cells.** To explore the effects of the soluble factors, including outer membrane vesicles, secreted by *N. meningitidis* on cell cycle alteration, Detroit 562 cells were next treated with bacterial supernatants of the different meningococcal isolates. Bacterial supernatants were collected from overnight cultures, followed by protein concentration (see Materials and Methods). Detroit 562 cells were exposed to viable *N. meningitidis* MC58 or 25- and 100-μl portions of meningococcal supernatants, correlating to 28.3 and 113.0 μg of protein, respectively. Although live bacteria induced an accumulation of cells in the G<sub>1</sub> phase as demonstrated before, meningococcal supernatants of all four *N. meningitidis* isolates had no effect on the cell cycle distribution (Fig. 5). A similar amount of cells in G<sub>1</sub> phase, S phase, and G<sub>2</sub>/M phase was observed when cells were either left untreated (control cells) or treated with bacterial supernatants of MC58, 8013/clone12, α711, or α4 (Fig. 5). These data suggest that it seems likely that continuous bacterial presence is necessary.

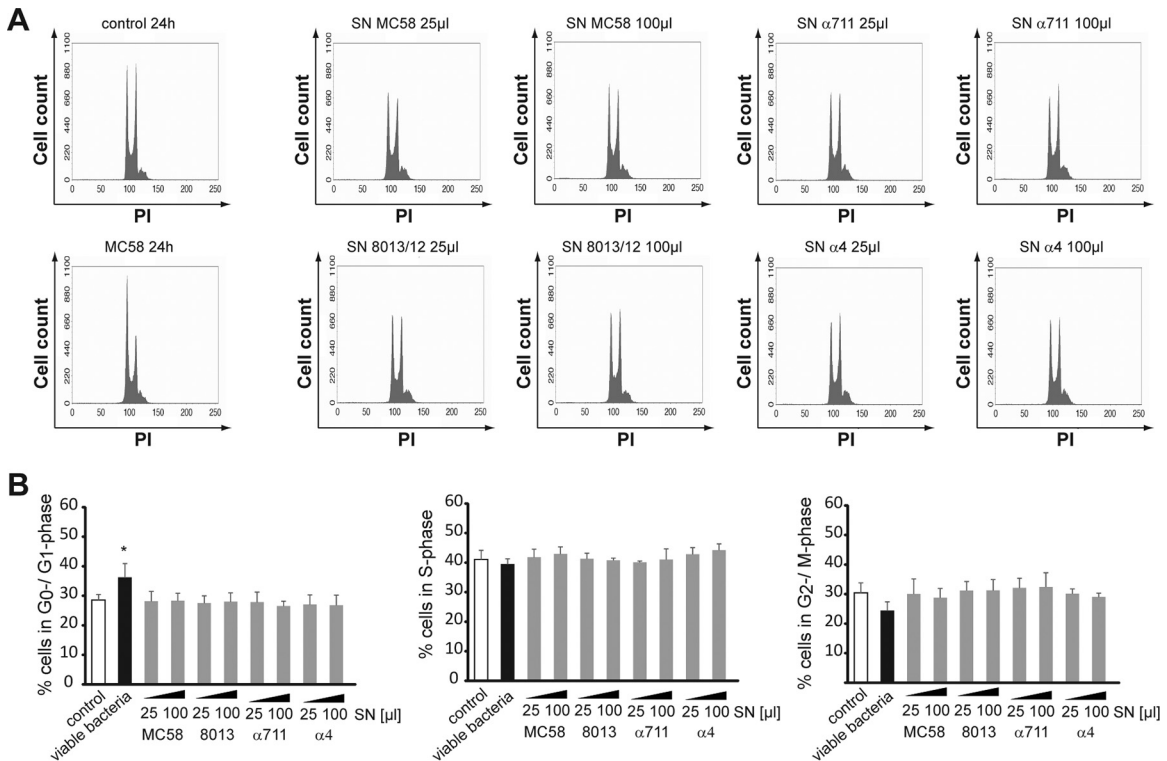
***N. meningitidis* infection affects cyclin D1 and cyclin E levels in the epithelial cell line Detroit 562.** Alterations of the cell cycle are predicted to result in changes in the abundance of the major cyclins, which, when complexed with a specific cyclin-dependent kinase (CDK), are one of the main drivers of cell cycle progression. Since the appropriate temporal activation of cyclin D/cdk4/6 and cyclin E/cdk2 is required for progression through the G<sub>1</sub> to the S phase (42–44), we investigated whether the observed accumulation of cells in the G<sub>1</sub> phase was reflected by a change in the abundance of these cyclins by analyzing protein expression levels by immunoblot analysis. Asynchronously growing Detroit 562 cells were infected with *N. meningitidis* at an MOI of 100 for a 24-h time period, cell lysates were collected and tested for cyclin D1 and cyclin E protein levels. After chemiluminescence band analysis, each protein signal was normalized to β-actin, and the changes were calculated (*n*-fold) between *N. meningitidis*-infected cells and uninfected control cells. The data indicated that the abundance of cyclin D1 significantly changed in Detroit 562 cells infected with carrier strains: cells infected with isolates α711 and α4 showed relative decreases by 0.4 and 0.2, respectively, in cyclin D1 expression at 24 h p.i. (*P* < 0.05), whereas cells infected with disease isolates 8013/12 and MC58 showed no significant changes in cyclin D1 levels (Fig. 6A). In contrast to cyclin D1 protein levels, *N. meningitidis* infection induced an increase in the levels of cyclin E at 24 h p.i. in response to all tested isolates: infected cells revealed 2.5-fold (MC58), 3.1-fold (8013/clone12), 2.7-fold (α711), and 1.8-fold (α4) increases, respectively, compared to uninfected control cells (Fig. 6A).



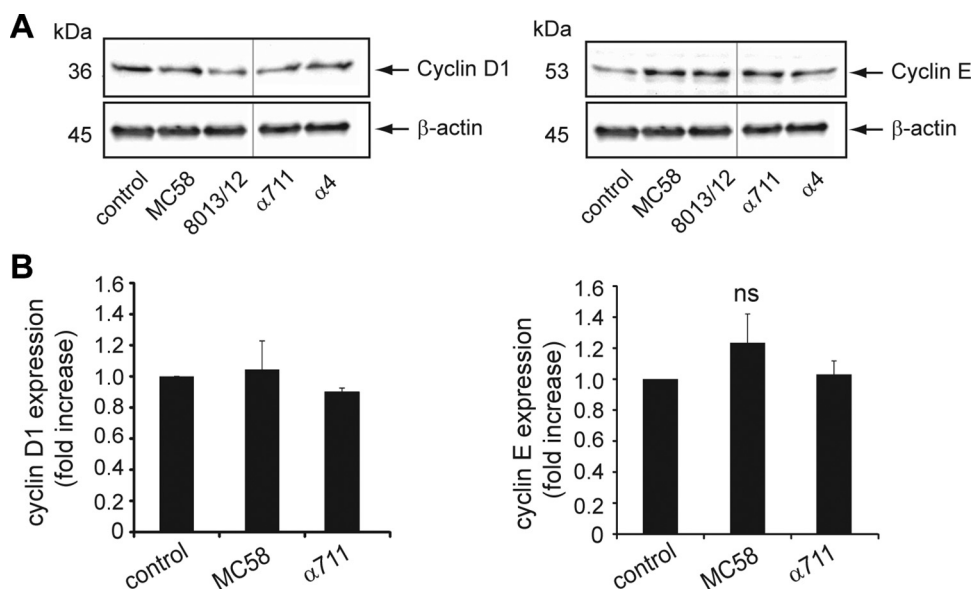
**FIG 4** G<sub>1</sub>-phase arrest is induced by viable bacteria but not by heat-killed bacteria. Detroit 562 cells either were infected with viable *N. meningitidis* strain MC58, 8013/clone12, α711, or α4 (MOI 100) or were exposed to heat-killed (hk) bacteria. Uninfected cells served as a control. Cells were harvested for PI staining at 24 h p.i. and analyzed by flow cytometry. (A) The results of representative experiments are shown. (B) The results of statistical analyses of the percentages of the cell population in the G<sub>1</sub>, S, and G<sub>2</sub>/M phases are shown. The data are shown as means ± the SD obtained from three independent experiments (\*, *P* < 0.05).

**Accumulation of cyclin E and loss of cyclin D1 expression are not controlled at transcriptional level.** To address the mechanism by which *N. meningitidis* induced cyclin E accumulation, we examined the levels of cyclin E mRNA at 24 h after infection of Detroit 562 cells with MC58 and carrier isolate α711. In parallel,

we determined the levels of cyclin D1 mRNA in response to the two isolates (Fig. 6B). No differences in the cyclin E and cyclin D1 mRNA levels were observed between the cells exposed to meningococcal isolates or uninfected control cells. This result confirmed that *N. meningitidis* does not induce the synthesis of cyclin E at a



**FIG 5** Meningococcal supernatants do not have any effect on the cell cycle distribution in epithelial cells. *N. meningitidis* strain MC58, 8013/clone 12, α711, or α4 was grown to the exponential phase and harvested by centrifugation, and supernatants were collected, filter sterilized, and transferred to VivaSpin columns for protein concentration. Detroit 562 cells either were left uninfected or were infected with viable MC58 (as a positive control) or were exposed to 25- or 100-µl portions of concentrated meningococcal supernatants. Cells were harvested for PI staining at 24 h p.i. and analyzed by flow cytometry. (A) The results of representative experiments are shown. (B) The graphs show the statistical analyses of the percentages of the cell population in the G<sub>1</sub>, S, and G<sub>2</sub>/M phases. The data are shown as means ± the SD obtained from three independent experiments (\*, *P* < 0.05).



**FIG 6** Pathogenic *N. meningitidis* strains and carrier isolates influence cyclin D1 and cyclin E protein levels. (A) Detroit 562 cells were either left uninfected (control) or infected with *N. meningitidis* MC58, 8013/clone 12, α711, or α4 (MOI 100) for 24 h. Cell lysates of control and *N. meningitidis*-infected cells were collected and analyzed for cyclin D1 and cyclin E protein expression by immunoblot analysis. β-Actin was used to normalize protein loading. The figure shows a representative Western blot with lanes from different areas of the same blot. (B) Bar diagram showing the fold increase in the normalized mRNA expression of cyclin D1 and cyclin E in Detroit 562 cells infected with MC58 or α711 in comparison to uninfected control cells. Real-time PCR data were analyzed according to the comparative  $\Delta C_T$  method by first normalizing the sample values (cyclin D1 and cyclin E) to the reference gene values (GAPDH) in infected and uninfected control cells, respectively, and then calculating the relative change in expression (as a fold increase) in infected compared to uninfected cells. Experiments were performed three times, and error bars represent the standard errors of the mean (ns, not significant).

transcriptional level. Increases in cyclin E protein levels are believed to be mainly regulated transcriptionally, although other control mechanisms have been described, including mRNA stabilization or prevention of protein degradation. In particular, transcription factor E2F-1 stabilizes cyclin E protein by preventing its ubiquitination, preserving the ability of the cyclin to bind and activate its kinase moiety (45).

***N. meningitidis* infection results in an accumulation of the CKI p21<sup>WAF1/CIP1</sup>.** The activities of cyclins and cyclin-CDK complexes are regulated by various mechanisms, including inhibition by CDK inhibitors (CKIs). Among them, the CKIs p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup>, both members of the kinase inhibitor proteins (KIPs), inhibit G<sub>1</sub>-specific CDK complexes and negatively regulate cellular proliferation (46, 47). When complexed with their respective cyclin binding partners, p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup> are able to block the kinase activity of CDKs (48). To analyze whether the observed cell cycle arrest in Detroit 562 cells treated with different meningococcal isolates was in correlation with altered activity of the CKI p21<sup>WAF1/CIP1</sup> or p27<sup>CIP1</sup>, asynchronously growing Detroit 562 cells were infected with both the pathogenic and the carrier isolates at an MOI of 100 for a 24-h time period, and cell lysates were subjected to immunoblot analysis to determine the abundance of p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup>. As shown in Fig. 7A, an increase of p21<sup>WAF1/CIP1</sup> total protein levels was observed in the infected cells in response to all *N. meningitidis* strains, reaching significantly higher values for MC58 and α711 (3.2- and 2.9-fold increases relative to the uninfected control, respectively). Unfortunately, we failed to detect p27<sup>CIP1</sup> in whole-cell lysates from infected Detroit 562 cells due to technical issues (data not shown).

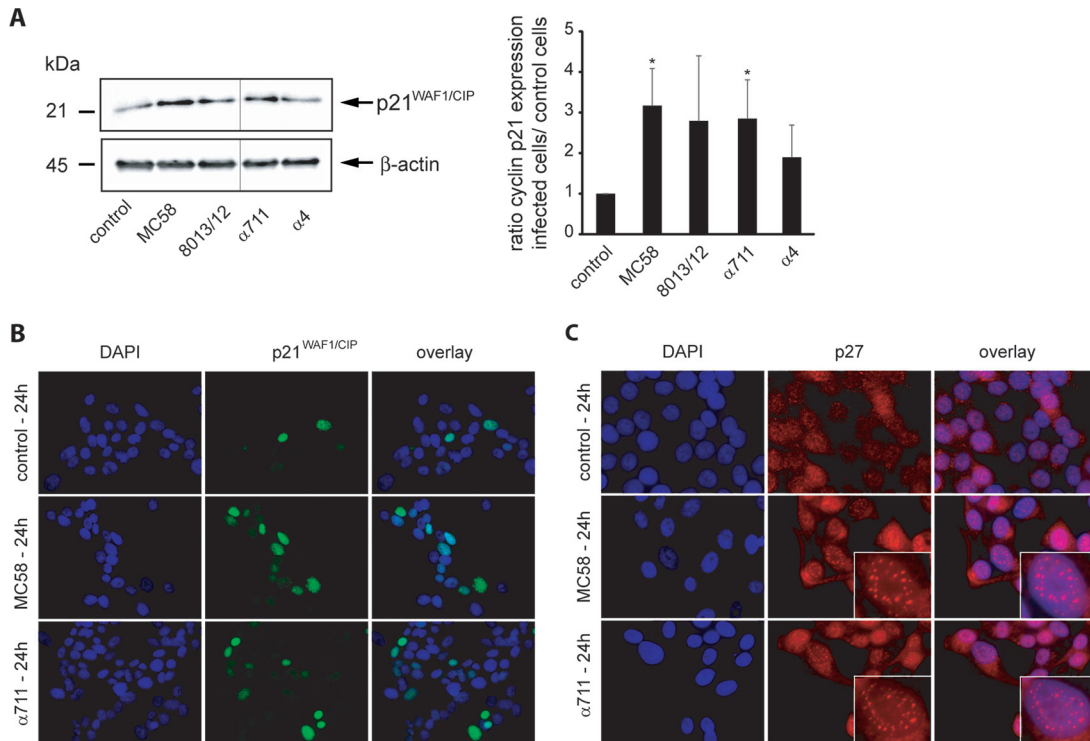
***N. meningitidis* infection results in a subcellular redistribution of p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup>.** We next investigated the sub-

cellular localization of p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup> in response to *N. meningitidis* infection by immunofluorescence microscopy. In infected Detroit 562 cells, strong nuclear accumulation of p21<sup>WAF1/CIP1</sup> was observed: 4/100 cells were positive for p21<sup>WAF1/CIP1</sup> nuclear staining in control cells versus 16/100 cells in MC58-infected cells at 24 h p.i. (Fig. 7B). These experiments indicated that in Detroit 562 cells, upon meningococcal infection, p21<sup>WAF1/CIP1</sup> accumulated in nuclei. Such an accumulation is in agreement with the function of p21<sup>WAF1/CIP1</sup> in the regulation of the cell cycle. Of note, much less profound accumulation of p21<sup>WAF1/CIP1</sup> was seen in the cytoplasm (Fig. 7B). Similar to p21<sup>WAF1/CIP1</sup>, p27<sup>CIP1</sup> needs to be transported into the nucleus to exert its inhibitory action. In noninfected cells, p27<sup>CIP1</sup> was visible inside the nucleus; however, in infected cells, staining revealed punctate p27<sup>CIP1</sup> foci in cells exposed to meningococci (Fig. 7C).

## DISCUSSION

Like many viruses that perturb the cell cycle machinery to adapt their own replication, bacteria can hijack checkpoints of the cell cycle to establish infection (1, 2, 8, 9). During microbial pathogenesis, the particular function to induce cell cycle arrest might represent a strategy to prevent maturation and exfoliation of epithelial cells to support prolonged bacterial persistence and survival in the epithelial cells. The concept that cell cycle modulation might favor bacterial colonization was demonstrated for the gut invasive pathogen *Shigella* (49) and for *Helicobacter pylori* that has adapted to the inhospitable conditions found at the gastric mucosal surface (50). The epithelium of the upper respiratory tract is classified as pseudostratified columnar epithelium; the epithelial cells closest to the basal lamina also undergo continuous mitosis, and their progeny replace the surface cells. Therefore, interference with cell





**FIG 7** Pathogenic *N. meningitidis* strains and carrier isolates increase p21<sup>WAF1/CIP1</sup> protein levels in Detroit 562 cells and induce redistribution of p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup>. (A) Cells were either left uninfected (control) or infected with *N. meningitidis* MC58, 8013/clone12, α711, or α4 (MOI 100) for a 24-h time period and were analyzed for p21<sup>WAF1/CIP1</sup> protein expression by Western blot analysis. Band intensities were quantified by densitometric analysis as described for cyclin D1 and cyclin E using ImageJ and normalized to β-actin. The figure shows a representative Western blot with lanes from different areas of the same blot. (B and C) Subcellular distribution of p21<sup>WAF1/CIP1</sup> (B) and p27<sup>CIP1</sup> (C) in *N. meningitidis*-infected Detroit 562 cells assessed by immunofluorescence microscopy. Colors in panel B: green, p21<sup>WAF1/CIP1</sup> protein stained with specific antibody and secondary antibody conjugated with Alexa Fluor 488; blue, nuclei stained with DAPI; light green, colocalization. Colors in panel C: red, p27<sup>CIP1</sup> stained with specific antibody and secondary antibody conjugated with Cy3; blue, nuclei stained with DAPI.

cycle progression might be an advantage for a bacterial pathogen that adheres to the epithelium of the upper respiratory tract to prolong its colonization in this particular niche. In the present study, we therefore investigated the effects of pathogenic *N. meningitidis* strains, as well as carrier isolates, on the cell cycle of the epithelial host cell. We demonstrate that both disease and carrier isolates can induce a G<sub>1</sub> arrest in the epithelial cell line Detroit 562 at 24 h p.i., which was paralleled by a significant decrease of cells in the S phase. We show that G<sub>1</sub> arrest was only induced after infection with live bacteria but not after infection with heat-killed bacteria. Bacterial infection with three of the tested meningococcal isolates resulted in a decreased protein level of cyclin D1, whereas cyclin E expression levels were increased in response to all meningococcal isolates. Furthermore, *N. meningitidis* infection induced an accumulation of the cyclin-dependent kinase inhibitor (CKI) p21<sup>WAF1/CIP1</sup> that was accompanied by a redistribution to the cell nucleus. In addition, the p27<sup>CIP1</sup> CKI was redistributed and showed punctate foci in infected cells.

In order to implement a suitable model for investigation of cell cycle alteration during meningococcal infection, we chose Detroit 562 cells, a pharyngeal epithelial carcinoma cell line, and NP69 cells, a nasopharyngeal epithelial cell line harboring *SV40T* (34). Detroit 562 cells are commonly used for *N. meningitidis* host cell interaction studies and exhibit high levels of bacterial adhesion (51–53). This cell line has been shown to express high levels of

carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a major Opa receptor, after exposure to cytokines or when infected with Opa-expressing *N. meningitidis* (52). NP69 cells were established from primary nonmalignant nasopharyngeal epithelial cells and may represent a model of premalignant nasopharyngeal epithelial cells (34, 54). The cell line was used to study Epstein-Barr virus interactions with the host cell (54, 55).

Both cell lines demonstrated equivalent data of bacterial adhesion when infected with the pathogenic meningococcal strain MC58. Moreover, the meningococcal wild-type strain MC58 elicited a time-dependent increase of uptake. In addition, the isogenic unencapsulated mutant showed a significantly higher rate of invasion compared to the wild-type strain due to the inhibitory effect of the serogroup B capsule polysaccharide of strain MC58 to bacterial invasion (22, 23, 32, 51, 56). We observed that meningococcal disease strain 8013/clone12 and carrier isolate α711 effectively adhered to Detroit 562 cells, whereas they were poorly invasive for both epithelial cell lines. In addition, low invasion rates were detected for carrier isolate α4, most probably due to low adhesion rates. For colonization of the human nasopharynx, the microorganism must adhere to the mucosal surface, utilize locally available nutrients, and evade the human immune system. For effective adhesion and invasion, *N. meningitidis* produces a number of structures and molecules, including the type IV pili, the outer membrane proteins Opa and Opc, and a number of newly

identified minor adhesion or adhesion-like proteins (21–28). For our study, we chose *N. meningitidis* MC58 as a prototype ST-32 cc strain and strain  $\alpha$ 711 as a genetically related carrier isolate from the ST-32 cc. We also included *N. meningitidis* isolate 8013/clone 12 from the ST-18 cc (also known as clone 12 or 2C43), which has been used to study the interaction between *N. meningitidis* and brain endothelial cells (57–60). Isolate  $\alpha$ 4 was chosen as a genetically related carrier isolate to 8013/clone 12. To decipher whether the poor invasive bacterial phenotype of the two carrier isolates and isolate 8013/clone 12 was due to differential expression of known adhesins or invasins, we characterized the tested isolates for expression of some adhesins/invasins, including the pili, Opc, and Opa. All isolates showed reactivity with the pilin antiserum and three expressed Opa proteins. However, all less invasive isolates did not express the Opc protein, either due to a deletion of the *opc* gene (8013/clone12 and  $\alpha$ 4) or as a result of transcriptionally regulated phase variation ( $\alpha$ 711), which is mediated by a variable polycytidine stretch in the promoter region of the gene (41). The contribution of Opc to mediate efficient uptake by epithelial and endothelial cells has been shown in previous studies (22, 23) and seems to be assignable for Detroit 562 and NP69 cells.

To establish colonization, bacteria have to counteract epithelial cell turnover. The nasal epithelium, as well as the mucosa of the extranasal upper respiratory tract, is in a steady state of cell renewal in which cell production balances cell loss (61). Epithelial cell proliferation is controlled either intracellularly via cell cycle regulatory factors, including cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CKIs), or retinoblastoma protein (pRb), or by extracellular factors (growth and differentiation factors). During the last decade, it became evident that bacteria have virulence mechanisms that target the cell cycle. To determine whether *N. meningitidis* is also able to impact the host cell cycle of epithelial cells, we determined the DNA profiles of PI-stained infected Detroit 562 cells. These data demonstrated that infected cells showed a significant accumulation of cells in the 2n DNA peak at 24 h p.i., suggesting an arrest or block of infected cells in the G<sub>1</sub> phase. In parallel, PI staining data could be verified by analyzing cells that were allowed to incorporate EdU and were simultaneously stained for DNA content. Moreover, we could show that only viable bacteria are capable to arrest epithelial cells in G<sub>1</sub> phase, whereas heat-killed bacteria had no effect. This suggested that the accumulation of cells in G<sub>1</sub> phase is most likely induced by a heat-labile bacterial factor or factors. Likewise, accumulation of cells in G<sub>1</sub> phase might be a result of a lower number of internalized bacteria that would be able to initiate various signaling cascades.

However, treatment with bacterial supernatants did not alter cell cycle distribution. Therefore, it seems likely that a continuous bacterial presence is necessary. Interestingly, *N. meningitidis* differently impacts the cell cycle depending on the cell type. Whereas in epithelial cells, meningococci induce a G<sub>1</sub> cell cycle arrest, we recently showed that *N. meningitidis* induces an accumulation of cells in the S phase of brain endothelial cells. Moreover, cell cycle alterations in brain endothelial cells could be assigned to the outer membrane proteins (Opa and the Opc protein) of *N. meningitidis* (31).

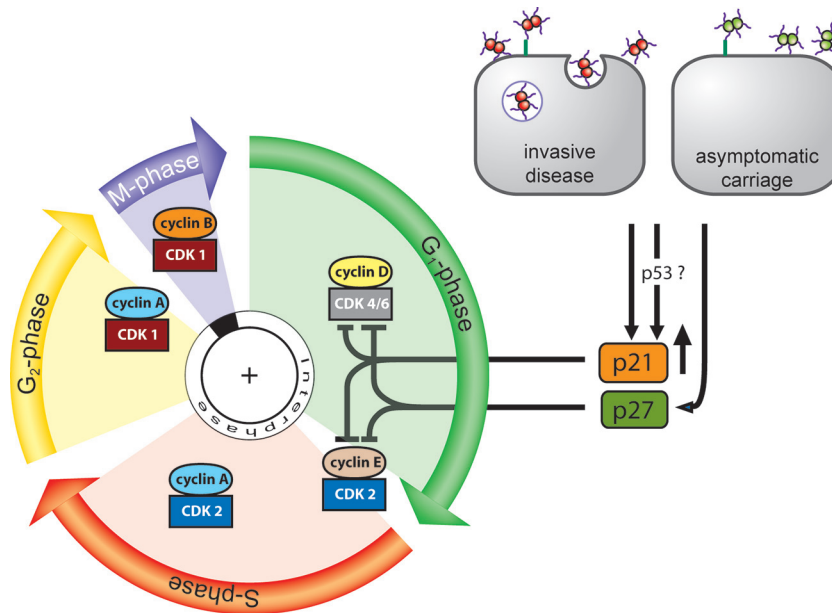
Progression through the cell cycle is driven by the activities of CDKs, in association with their regulatory subunits, the cyclins (43, 44). In particular, type D and E cyclins mediate G<sub>1</sub>- to S-phase cell cycle progression through the activation of specific CDKs that

phosphorylate pRb, thus inactivating it and releasing the transcription factor E2F from its inhibition. E2F then activates a number of growth-promoting genes, which drive the cell cycle into the S phase. Whereas D-type cyclins associate with cdk4 and cdk6, cyclin E associates with cdk2. Our studies showed that cyclin D1 protein levels were partially decreased in response to meningococcal isolates, whereas the cyclin D1 mRNA levels were not changed in infected cells. Since the levels of cyclins are regulated at the level of transcription or by targeted degradation via the ubiquitin pathway (62), our data suggest that the decrease in the cyclin D1 protein levels during meningococcal infection was most likely due to proteasomal degradation. In addition, our data showed that cyclin E protein levels increased at 24 h p.i. in response to all meningococcal strains, whereas the mRNA levels in infected cells were comparable to those in uninfected cells, suggesting that increased cyclin E protein levels were not due to increase of gene transcription. Whether inhibition of cyclin E ubiquitination and proteasomal degradation occurs during meningococcal infection is questionable and remains to be elucidated.

Our data furthermore clearly indicated a significant increase of p21<sup>WAF1/CIP1</sup> protein levels. p21<sup>WAF1/CIP1</sup> is the archetypal mammalian CKI and is tightly controlled by the tumor suppressor protein p53 through which this protein mediates the p53-dependent cell cycle G<sub>1</sub>-phase arrest in response to a variety of stress stimuli. In addition, it can be induced by p53-independent mechanisms. p21<sup>WAF1/CIP1</sup> can bind to cyclinD-cdk4 or cyclinD-cdk6 complexes and prevents them from phosphorylating pRb. Moreover, cyclinE-cdk2 complexes can be negatively regulated by the CKI p21<sup>WAF1/CIP1</sup>. Similar to p21<sup>WAF1/CIP1</sup>, p27<sup>CIP1</sup> can inhibit cyclin-CDK complexes in G<sub>1</sub> phase, and it is functional in the nucleus. Unfortunately, we failed to detect p27<sup>CIP1</sup> expression by immunoblotting in infected Detroit 562 cells due to technical issues. However, we could detect significant increase of punctate foci of p27<sup>CIP1</sup> in infected cells by immunofluorescence analysis. Such an accumulation as observed during meningococcal infection is in line with the function of p27<sup>CIP1</sup> in the regulation of the cell cycle. p27<sup>CIP1</sup> expression is regulated at different levels, i.e., transcriptional, translational, and posttranslational mechanisms, including ubiquitin-proteasome-induced degradation, as well as protein phosphorylation. Taken together, we would assume that the downstream mechanisms of *N. meningitidis*-induced G<sub>1</sub> cell cycle arrest might involve p21<sup>WAF1/CIP1</sup> recruitment to cyclinD-cdk4/6 complexes and/or cyclin E-cdk2 complexes, thereby inhibiting CDK activity and thus the phosphorylation of pRb (Fig. 8). However, future studies have to clarify the exact mechanism by which an accumulation of p21<sup>WAF1/CIP1</sup> results in G<sub>1</sub> arrest in epithelial cells in response to meningococcal infection.

During the past decade, several bacterial effectors have been shown to interfere with p21<sup>WAF1/CIP1</sup> expression, such as the cyclomodulin cytolethal distending toxin from *E. coli* (63, 64), toxin A from *Clostridium difficile* (65), or the CagA effector from *Helicobacter pylori* (66–68). Interestingly, the cycle inhibiting factor (Cif) produced by EPEC and EHEC bacteria differs from the toxins mentioned above in that Cif-induced accumulation of p21<sup>WAF1/CIP1</sup> does not involve transcription mechanisms but acts on a pathway controlling protein stability (69).

*N. meningitidis* can asymptotically colonize the upper respiratory tract of healthy carriers for months or years (70). The asymptomatic carriage of *N. meningitidis* is of concern since it is the mechanism by which the reservoir of endemic meningococci is



**FIG 8** Proposed model of G<sub>1</sub> cell cycle arrest in epithelial cells during meningococcal infection. Infection of pharyngeal epithelial cells with both invasive meningococcal disease isolates and apathogenic carriage isolates results in increased protein levels of the CKI p21<sup>WAF1/CIP1</sup> and nuclear redistribution of p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup>. Increased p21<sup>WAF1/CIP1</sup> levels may be required for inhibition of the G<sub>1</sub>-phase complexes cyclin D-cdk4/6 and/or cyclin E-cdk2 by one or both of the CKIs, resulting in subsequent sequestration of cells in the G<sub>1</sub> phase. G<sub>1</sub>-phase, gap phase 1; S-phase, DNA synthesis; G<sub>2</sub>-phase, gap phase 2; M-phase, mitosis; CDK, cyclin-dependent kinase.

maintained within the population. Adaptation to this niche not only requires mechanisms to effectively adhere to and invade the host cells but also includes mechanisms that help to counteract epithelial cell turnover. In this study, we now provide evidence that both disease and carrier isolates of *N. meningitidis* can interfere with cell cycle progression by sequestration of targeted host cells in G<sub>1</sub>. The cell cycle arrest induced by *N. meningitidis* may represent a strategy to prevent maturation and exfoliation of epithelial cells to support prolonged bacterial persistence and survival in the epithelial cells of the upper respiratory tract. However, further investigations are needed to monitor the attachment of meningococci to epithelial cells that have been arrested in the G<sub>1</sub> phase. Interestingly, the related species *N. gonorrhoeae* has also been shown to modulate the host cell cycle (71, 72). In line with our findings, *N. gonorrhoeae* also arrests cells in the early G<sub>1</sub> phase of the cell cycle after 24 h of infection, as shown for unsynchronized HeLa cells (71). In a follow-up study, the authors could show that the accumulation of infected cells in G<sub>1</sub> was a result of a delay in the G<sub>2</sub>-to-M-phase progression and the sequestration of cells in G<sub>2</sub> (72). Mechanistically, the sequestration of cells in G<sub>2</sub> was a result of increased expression of p21<sup>WAF1/CIP1</sup> and p27<sup>CIP1</sup>. Relevant to our study, neither supernatants from infected cell cultures nor bacterial supernatants had any effect on the cell cycle. Although research in the last decade has shown that pathogenic bacteria can interfere with host cell cycle progression (1, 2, 8), one of the open questions remained whether commensal bacteria also produce “cyclomodulins” and whether commensal bacteria can interfere with epithelial cell turnover. Here, we now provide evidence that carrier isolates of the species *Neisseria* can also impact on the host cell cycle. Future studies are necessary to identify the molecular components of the meningococci responsible for eliciting cell cycle arrest in epithelial cells. Since both the rate of cell

proliferation and cell migration contribute to the turnover time of the respiratory epithelium, future studies will also rely on more complex cell culture models, for example, the established three-dimensional organotypic tissues of the human mucosa, to achieve a full understanding of the interference and control of the cell cycle during bacterial pathogenesis.

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