NlpI Facilitates Deposition of C4bp on *Escherichia coli* by Blocking Classical Complement-Mediated Killing, Which Results in High-Level Bacteremia

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Neonatal meningitis *Escherichia coli* (NMEC) is the most common Gram-negative organism that is associated with neonatal meningitis, which usually develops as a result of hematogenous spread of the bacteria. There are two key pathogenesis processes for NMEC to penetrate into the brain, the essential step for the development of *E. coli* meningitis: a high-level bacteremia and traversal of the blood-brain barrier (BBB). Our previous study has shown that the bacterial outer membrane protein NlpI contributes to NMEC binding to and invasion of brain microvascular endothelial cells, the major component cells of the BBB, suggesting a role for NlpI in NMEC crossing of the BBB. In this study, we showed that NlpI is involved in inducing a high level of bacteremia. In addition, NlpI contributed to the recruitment of the complement regulator C4bp to the surface of NMEC to evade serum killing, which is mediated by the classical complement pathway. NlpI may be involved in the interaction between C4bp and OmpA, which is an outer membrane protein that directly interacts with C4bp on the bacterial surface. The involvement of NlpI in two key pathogenesis processes of NMEC meningitis may make this bacterial factor a potential target for prevention and therapy of *E. coli* meningitis.

Escherichia coli is the most common Gram-negative organism that causes neonatal bacteremia and meningitis (8, 29). *E. coli* meningitis, which usually develops as a consequence of hematogenous spread of the bacteria, requires a high degree of bacteremia for the pathogen to traverse the blood-brain barrier (BBB) and penetrate into the brain (14, 15). However, the microbial determinants contributing to neonatal meningitis *E. coli* (NMEC) survival in the blood and bacteremia remain incompletely understood.

The NlpI protein of *E. coli* has been shown to contribute to NMEC binding to and invasion of human brain microvascular endothelial cells (HBMECs), which constitute the BBB. This finding suggests the role of this protein in the traversal of NMEC through the BBB (35). NlpI has also been identified to contribute to the interaction between Crohn's disease-associated *E. coli* strain LF82 and intestine epithelial cells (1). NlpI is an outer membrane lipoprotein (24, 35). The *nlpI* mutant of *E. coli* exhibits a growth defect at 42°C in low-salt Luria-Bertani (LB) medium (24). In this study, we demonstrate that NlpI is involved in the high-level bacteremia caused by NMEC.

To cause a high-level bacteremia, NMEC must resist or escape the host immune system-mediated killing in the bloodstream. The complement system is an important part of innate immunity in the bloodstream and is involved in opsonization and lysis of invading bacterial pathogens. Activation of the complement system occurs through a sequence of enzymatic reactions that are triggered by three pathways, i.e., the classical, lectin, and alternative pathways (40). The classical complement pathway is mainly initiated by the binding of C1q to the Fc portion of an antibody-antigen complex on the bacterial surface. The lectin pathway is

primarily activated through the binding of mannose-binding lectin (MBL) to certain carbohydrate motifs on the bacterial cell surface. The alternative pathway is initiated by the spontaneous hydrolysis of the serum protein C3 to produce C3b. These pathways converge at the deposition of the C3 derivative, C3b, on target pathogens, which leads to opsonization of the target and activation of the common terminal complement pathway. The activation of the terminal complement pathway results in the formation of the membrane attack complex (MAC) on the bacterial surface, which causes bacteriolysis.

Host regulatory proteins can inhibit the excessive activation of the complement system to protect the host from complement attack, but this strategy can be adopted by pathogens to evade complement-mediated killing. For example, the C4b-binding protein (C4bp) is the major inhibitor of the classical and lectin pathways. The C3 convertase, C4bC2a, is derived from the activation of these two pathways and catalyzes C3b production. C4bp interacts with C4b to interfere with the formation of C4bC2a and thus hinders the formation of C3b that results from the activation of these two

Received 14 April 2012 Returned for modification 20 May 2012

Accepted 8 July 2012

Published ahead of print 16 July 2012

Editor: S. M. Payne

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Supplemental material for this article may be found at <http://iai.asm.org/>.

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doi:10.1128/IAI.00320-12

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant information	Sources or reference(s)
Strains		
RS218 (WT)	NMEC RS218 isolated from the CSF of a neonate with meningitis	35, 42
RS218 $\Delta nlpI$	RS218 mutant with an <i>nlpI</i> deletion	35
RS218 $\Delta ompA$	RS218 mutant with an <i>ompA</i> deletion	35
RS218 $\Delta nlpI \Delta ompA$	RS218 mutant with an <i>nlpI</i> and <i>ompA</i> double deletion	35
L346	RS218 with a <i>lacZ</i> deletion	40a
Plasmids		
pCL1920	Low-copy-number plasmid	18, 32
pNI1	pCL1920 harboring the <i>nlpI</i> gene and its promoter region	32
pN10-His	pTrc99A harboring a DNA fragment encoding C-terminal His ₆ -tagged NlpI	35
pET28a-NlpI	pET28a harboring a DNA fragment encoding N-terminal His ₆ -tagged NlpI	This study

pathways (5–7). Many bacterial pathogens, such as NMEC, *Streptococcus pyogenes*, *Bordetella pertussis*, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, have been found to recruit the host C4bp to their surfaces to escape from complement-mediated killing (2, 9, 12, 22, 28, 38).

In this study, we report a novel role of NlpI in *E. coli* bacteremia. This protein is critical for NMEC evasion of the host complement system. Our results demonstrated that NlpI was involved in the deposition of C4bp on the surface of bacteria to avoid serum killing mediated by the classical complement system. This finding may explain the basis for the decreased ability of the NMEC *nlpI* mutant to cause a high-level bacteremia.

MATERIALS AND METHODS

Bacterial strains. The NMEC strain RS218 (O18:K1:H7) was isolated from the cerebrospinal fluid (CSF) of a neonate with meningitis (31). In this study, the spontaneous, streptomycin-resistant derivative of RS218 was designated the wild-type RS218 strain (WT RS218), and its derivatives were used (Table 1) (35, 42).

Human serum and purified C4bp. Human sera were obtained from 10 healthy adult volunteers and were pooled, aliquoted, and stored at -80°C . The pooled serum was designated normal human serum (NHS). Heat-inactivated NHS (HI-NHS) was prepared by incubating the NHS at 56°C for 30 min. Purified C4bp was purchased from Complement Technology, Inc. (Tyler, TX). The procedures for collecting human serum were approved by the Institutional Reviewer Board (IRB) of National Cheng Kung University Hospital, Tainan City, Taiwan.

Preparation of C4bp-depleted serum. Approximately 10 mg of sheep, anti-C4bp IgG (Abcam Inc., Cambridge, MA) was coupled to a HisTrap, NHS-activated column (1 ml; GE Healthcare Bio-Science Corp., Piscataway, NJ) following the manufacturer's instructions. To prepare C4bp-depleted NHS, 40% NHS (diluted in phosphate-buffered saline [PBS]) was passed through this column by syringe injection at room temperature. The control NHS was prepared in the same manner that was used for the C4bp-depleted NHS using a HisTrap, NHS-activated column, but no anti-C4bp IgG coupling was performed. The C4bp levels in the resulting sera were measured by Western blot analysis with the anti-C4bp antibody (see Fig. S1 in the supplemental material).

Modification of NHS. To inhibit the alternative pathway, NHS was depleted of properdin with 10 mg/ml of bentonite as described previously (13). Briefly, 40% NHS (diluted in PBS) was incubated with bentonite at 37°C for 10 min. The serum was centrifuged to remove the bentonite and filter sterilized. The lectin pathway was specifically inhibited with 100 mM mannose as previously described (19, 25); goat anti-C1q serum (Calbiochem, Darmstadt, Germany) was used to inhibit the classical pathway as previously described (10). To inhibit the classical pathway of NHS, heat-

inactivated C1q antiserum was added to a final concentration of 10% (vol/vol) in the 40% NHS (diluted in PBS).

Serum survival assay. Log-phase bacteria (1×10^5 CFU) were incubated in 100 μl of 40% NHS (or modified NHS; diluted in PBS) at 37°C . At various times, 10- μl aliquots were collected, and each sample was spread onto an LB agar plate. After culturing overnight, the bacterial numbers were counted. Bacteria incubated in 40% heat-inactivated NHS were included as a control. The survival rates were calculated by the ratio of the bacterial counts to those of the original inocula at different time points.

LIVE/DEAD staining of bacteria. To determine the ratio of live and dead bacterial cells, bacteria were subjected to LIVE/DEAD staining using a LIVE/DEAD BacLight kit (Molecular Probes, Eugene, OR), following the manufacturer's instruction, after the bacteria were incubated with 40% NHS for 120 min. By immunofluorescence microscopy analyses, live cells were stained by Syto9 to be green labeled, while dead cells were stained by propidium iodide to be red labeled.

Flow cytometry analysis. To determine the deposition of C1q, MAC, and C4bp on *E. coli*, 1×10^5 CFU of log-phase bacteria were incubated in 100 μl of 60% human serum that was diluted with Veronal buffer (Lonza Walkersville, Inc., Walkersville, MD) at 37°C for different periods. After 3 washings, the bacteria were incubated with antibodies against C1q (Calbiochem, Darmstadt, Germany), MAC (Abcam Inc., Cambridge, MA), or C4bp (Abcam) at room temperature for 30 min; and 3 washings with PBS followed these incubations. The primary antibody-labeled bacteria were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 30 min at room temperature and subsequently washed 3 times with PBS. To determine the deposition of C3b, the FITC-conjugated anti-C3 antibody (Abcam) was used by following the above-mentioned protocol without secondary antibody treatment. The surface depositions of the molecules were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

PMN killing assay. Log-phase bacteria (5×10^5 CFU) were incubated with 5×10^5 polymorphonuclear leukocytes (PMNs) in 1 ml of Dulbecco modified Eagle medium that contained either 10% NHS, 10% HI-NHS, or no serum, as described previously (16). The mixtures were rotated end over end for 60 min at 37°C , and the bacterial numbers were enumerated.

Neonatal rat model of *E. coli* bacteremia. *E. coli* bacteremia was induced in 5-day-old rats by subcutaneous injection of *E. coli* as previously described (11). Briefly, all members of each litter were randomly divided into the experimental groups to subcutaneously receive 2×10^5 CFU of *E. coli* strain RS218 or its *nlpI* mutant. At 18 h after bacterial inoculation, blood specimens were collected for quantitative cultures. The Johns Hopkins University Animal Care and Use Committee approved all procedures and handling techniques.

Immunofluorescence microscopy analysis. To determine the surface exposure of NlpI on *E. coli*, NMEC RS218 derivatives which harbored pNI10-His or pTrc99A were induced with 1 mM isopropyl- β -D-thio-

TABLE 2 Magnitude of bacteremia with RS218 $\Delta nlpI$ than WT RS218 in 5-day-old rats

Strain (no. of animals)	Mean bacteremia (log CFU/ml of blood) $\pm$ SD
WT RS218 (8)	8.05 $\pm$ 1.13
RS218 $\Delta nlpI$ (8)	3.21 $\pm$ 1.81 ^a
WT RS218/pCL1920 (5)	8.99 $\pm$ 3.07
RS218 $\Delta nlpI$ /pCL1920 (5)	4.65 $\pm$ 1.33 ^b
RS218 $\Delta nlpI$ /pNII ^c (6)	7.57 $\pm$ 2.12

^a Significantly lower ($P < 0.01$) than that for RS218 $\Delta nlpI$.^b Significantly lower ($P < 0.01$) than that for WT RS218/pCL1920 and RS218 $\Delta nlpI$ /pNII.^c pNII is a pCL1920-derived plasmid harboring the *nlpI* gene.

lactopyranoside (IPTG) for 2 h and fixed on poly-L-lysine-coated slides as previously described (33). The bacteria were subjected to immunofluorescence labeling with the anti-His₆ monoclonal antibody at a 1:50 dilution, followed by incubation with FITC-conjugated goat anti-mouse IgG antibodies at a 1:100 dilution at room temperature for 1 h. Then, the labeled bacteria were analyzed using an immunofluorescence microscope.

Overexpression and purification of N-terminally His₆-tagged NlpI and preparation of rabbit NlpI antiserum. The *nlpI* gene was amplified by PCR with the following primer pair: 5'-GGAATTCATATGAGTAA TACTTCCTGGCGTAAAAG-3' and 5'-CGGGATCCCTATTGCTGGT CCGATTCTGCCAGG-3'. The PCR product was doubly digested with NdeI and BamHI and subcloned into the overexpression vector pET28a (Novagen, San Diego, CA), which encoded a His₆ tag fused to the 5' end of the *nlpI* gene. The resulting plasmid was named pET28a-NlpI (Table 1). In the resulting recombinant NlpI, the 19 N-terminal residues of the NlpI polipoprotein were replaced with 21 vector-derived residues that contained a His₆ tag. To overexpress and purify the N-terminally His₆-tagged NlpI, *E. coli* strain BL21(DE3) (Novagene) transformed with pET28a-NlpI was induced with 1 mM IPTG for 2 h at 30°C, and then the overexpressed recombinant protein was purified by metal-chelate chromatography with an Ni-charged column as described previously (41). The purified recombinant protein was utilized to raise rabbit NlpI antiserum by following the procedures described previously (34). The antiserum was able

to recognize the recombinant protein and also detect NlpI in wild-type RS218 but not in its *nlpI* mutant (see Fig. S2 in the supplemental material).

Statistical analysis. The Student *t* test was used for statistical analysis. A *P* value of < 0.05 was used as the threshold for statistical significance.

RESULTS

The *nlpI* mutant of NMEC RS218 failed to induce a high degree of bacteremia. To determine the role of NlpI in induction of a high level of bacteremia, the critical step in the pathogenesis of NMEC meningitis, the wild-type (WT) NMEC strain RS218 and its *nlpI* mutant (RS218 $\Delta nlpI$) were subcutaneously injected into 5-day-old rats and blood specimens were collected at 18 h postinoculation to be used for bacterial counts. As shown in Table 2, RS218 $\Delta nlpI$ was significantly defective in inducing a high degree of bacteremia compared to WT RS218. Transcomplementation of the mutant with a plasmid harboring *nlpI* (pNII) restored the mutant's ability to induce a high level of bacteremia (Table 2), suggesting that the inability of RS218 $\Delta nlpI$ to induce a high degree of bacteremia was not derived from a polar effect caused by deletion of the *nlpI* gene. These findings indicate that NlpI is involved in inducing the high degree of bacteremia of NMEC.

The decreased survival in blood of the *nlpI* mutant of NMEC is not related to PMN-mediated killing. PMNs are part of the host innate immunity against invading pathogens. To investigate whether PMN-mediated killing is responsible for the decreased survival of the *nlpI* mutant in the bloodstream, PMN killing assays of WT RS218 and RS218 $\Delta nlpI$ were performed. In assays where PMN killing was done without NHS or NHS with 10% heat-inactivated serum (HI-NHS), WT RS218 and RS218 $\Delta nlpI$ exhibited similar levels of survival. However, in assays where 10% NHS was subjected to opsonization, RS218 $\Delta nlpI$ showed significantly decreased survival in comparison with the wild-type strain (Fig. 1A). The decreased survival of RS218 $\Delta nlpI$ was restored to the level of WT RS218 by transcomplementation of the mutant with pNII (Fig. 1B). These findings suggest that PMNs alone may not be responsible for the decreased survival of RS218 $\Delta nlpI$ in the blood-

FIG 1 Abilities of WT RS218 and RS218 $\Delta nlpI$ to resist PMN-mediated killing. (A) Survival of *E. coli* strains that were mixed with PMNs and incubated with 10% NHS, with 10% HI-NHS, or without serum for 60 min; (B) survival of RS218 $\Delta nlpI$ transformed with *nlpI*-harboring plasmid pNII or with the empty vector, pCL1920, and survival of WT RS218 containing pCL1920 after incubation with PMNs in 10% NHS for 60 min. The results are presented as the relative survival rates of WT RS218 (A) and WT RS218 harboring pCL1920 (B). The data shown are representative of three independent experiments which were performed in triplicate and which used PMNs from different individuals. The results are presented as the mean $\pm$ SD. *, $P < 0.05$.

FIG 2 Involvement of NlpI in the ability of NMEC to survive in human serum. (A) Survival of WT RS218 and RS218 $\Delta nlpI$ in NHS and HI-NHS; (B) survival of RS218 $\Delta nlpI$ transformed with *nlpI*-harboring plasmid pNI1 or with the empty vector, pCL1920, and survival of WT RS218 containing pCL1920 in NHS and HI-NHS; (C) survival of WT RS218 and RS218 $\Delta nlpI$ that were coincubated in NHS or HI-NHS; (D) survival of WT RS218 and RS218 $\Delta nlpI$ in HI-NHS at 41°C. The survival rates were calculated by the ratio of the bacterial counts to those of the original inocula at different time points. The data shown are representative of three independent experiments performed in triplicate. The results are presented as the mean $\pm$ SD.

stream and that differential levels of opsonization by the heat-labile components (e.g., complement [23]) are likely to occur and induce different levels of killing between WT RS218 and RS218 $\Delta nlpI$.

NlpI is involved in serum resistance of NMEC. To assess whether the decreased ability of RS218 $\Delta nlpI$ to induce a high degree of bacteremia was due to serum killing, the survival rates of RS218 $\Delta nlpI$ in NHS and HI-NHS were compared with those of WT RS218 (we use “survival rate” instead of “growth rate” in this study, because the RS218 $\Delta nlpI$ cells were significantly killed in NHS, according to the LIVE/DEAD staining assay of the bacteria after incubation with NHS [see Fig. S3 in the supplemental material]). The mutant exhibited a significantly lower ability to survive in NHS than the wild-type strain, but the wild-type and mutant strains showed similar survival rates in HI-NHS (Fig. 2A). In addition, in NHS, RS218 $\Delta nlpI$ transcomplemented with plasmid pNI1 showed a significantly higher level of survival than RS218 $\Delta nlpI$ harboring the control vector, pCL1920, and the survival of pNI1-complemented RS218 $\Delta nlpI$ was similar to that of WT RS218 harboring pCL1920 (Fig. 2B). These findings suggest that NlpI is likely to be involved in NMEC evasion of complement-mediated serum killing.

When coincubated with the otherwise wild-type strain L346 (the *lacZ*-deletion mutant of RS218), RS218 $\Delta nlpI$ still exhibited a lower survival rate in NHS (Fig. 2C), indicating that coexistence of a wild-type strain does not restore the ability of RS218 $\Delta nlpI$ to

survive in NHS. This result suggests that RS218 $\Delta nlpI$ is not likely to be deficient in producing or secreting into NHS a bacterial factor which is required for bacterial survival in serum.

The *nlpI* mutant of *E. coli* K-12 has been shown to be thermosensitive in low-salt LB medium but not in LB medium with a regular concentration of salt (24). RS218 $\Delta nlpI$ also showed a similar growth phenotype (data not shown). Then, we found that RS218 $\Delta nlpI$ exhibited survival similar to that of WT RS218 in HI-NHS at 41°C, suggesting that the mutant is not thermosensitive in human serum (Fig. 2D). Therefore, a high fever occurring in an infected host may not be a factor contributing to the host’s differential killing of WT RS218 and RS218 $\Delta nlpI$ in the bloodstream.

RS218 $\Delta nlpI$ exhibits higher levels of C3b and MAC deposition than WT RS218 in NHS. The levels of C3b or MAC deposition on bacterial cells signal the intensity of the complement activation that is triggered by the bacteria in NHS. To further determine whether NlpI was involved in NMEC evasion of complement-mediated serum killing, the levels of C3b and MAC deposition on RS218 $\Delta nlpI$ and WT RS218 were measured by flow cytometry after incubation with NHS. The amount of bacterial debris derived from serum-killed *E. coli* is likely to increase with the duration of NHS incubation with the bacteria. Therefore, to decrease the potential interference of bacterial debris in the flow cytometry analysis, we measured the complement deposition on bacteria after incubation in NHS for up to 60 min, although the

FIG 3 Deposition of C3b and MAC on WT RS218 and RS218 $\Delta nlpI$ after incubation in NHS for various periods. (A) Levels of C3b deposition on RS218 $\Delta nlpI$ determined by flow cytometry compared with those on WT RS218; (B) flow cytometry histogram of C3b deposition on the bacteria after a 60-min incubation in NHS; (C) levels of MAC deposition on RS218 $\Delta nlpI$ determined by flow cytometry compared with those on WT RS218; (D) flow cytometry histogram of MAC deposition on the bacteria after a 60-min incubation in NHS. The data of the flow cytometry analyses in panels A and C are presented as the relative mean fluorescent intensity (MFI) compared to the deposition on WT RS218. These quantitative data were derived from two independent experiments. *, $P < 0.05$.

magnitude of the difference in serum survival between the wild type and mutant was greater after a 120-min incubation in NHS than after a 60-min incubation (Fig. 2A). In agreement with the results of the serum survival assays shown in Fig. 2A, the levels of C3b and MAC deposition on RS218 $\Delta nlpI$ cells were significantly higher than those on WT RS218 cells after the 60-min incubation in NHS (Fig. 3A to D). These results demonstrate that NlpI is likely to be involved in NMEC evasion of the complement system.

NlpI is mainly involved in resistance of NMEC to serum killing mediated by the classical complement pathway. To investigate which of the three complement pathways are responsible for the differential killing of WT RS218 and RS218 $\Delta nlpI$, the survival rates of the two strains were examined in modified human serum (i.e., the classical, alternative, or lectin pathways were inhibited) (Fig. 4A to C). In the human serum with a blocked classical pathway (by treatment with heat-inactivated C1q antiserum), the survival rates of WT RS218 and RS218 $\Delta nlpI$ were not significantly different (Fig. 4A). In contrast, in the human serum with a blocked alternative pathway (by bentonite treatment) or a blocked lectin pathway (by mannose treatment), RS218 $\Delta nlpI$ still exhibited significantly lower survival than WT RS218 (Fig. 4B and C). These results suggest that NlpI is mainly involved in NMEC evasion of serum killing mediated by the classical complement pathway.

RS218 $\Delta nlpI$ is less able to recruit C4bp than WT RS218. The level of C1q deposition on the bacterial surface reflects the intensity of the initial activation of the classical complement pathway triggered by the bacteria in NHS. However, we found that the levels of C1q deposition on RS218 $\Delta nlpI$ and WT RS218 were not significantly different after a 10-, 30-, or 60-min incubation in NHS (data not shown). These results suggest that the involvement

of NlpI in bacterial evasion of the classical complement attack is not likely to occur through modulating the initial activation of this complement cascade in NHS.

Because NMEC is shown to be able to recruit C4bp onto its surface to suppress the activation of the classical pathway cascade downstream of C1q deposition (28), we investigated whether deletion of *nlpI* affects the ability of NMEC to recruit C4bp on the surface. The levels of C4bp deposition on RS218 $\Delta nlpI$ were significantly lower than those on WT RS218 after incubation in NHS for 30 and 60 min (Fig. 5A and B). Consistently, after incubation with purified C4bp protein for 60 min, WT RS218 recruited a significantly higher level of C4bp than RS218 $\Delta nlpI$ (Fig. 5C). These results suggest that NlpI is likely to be involved in the deposition of C4bp on the bacterial surface to facilitate bacterial evasion of the classical complement attack in NHS.

The decreased ability of RS218 $\Delta nlpI$ to recruit C4bp contributes to the mutant's decreased serum survival. To determine whether the decreased ability of RS218 $\Delta nlpI$ to recruit C4bp is responsible for its decreased ability to survive in human serum, we examined and compared the survival of WT RS218 and RS218 $\Delta nlpI$ in C4bp-depleted serum (Fig. 6). WT RS218 exhibited significantly lower survival in C4bp-depleted serum than in the control NHS, but the survival of the *nlpI* mutant in serum with or without C4bp was similar. In addition, supplementation of purified C4bp into C4bp-depleted serum restored the survival of WT RS218, but this treatment did not affect the survival of RS218 $\Delta nlpI$. These results indicate that, without *nlpI*, NMEC is not able to utilize C4bp to evade serum killing and demonstrate that decreased C4bp deposition on RS218 $\Delta nlpI$ contributes to this mutant's decreased ability to resist serum killing.

FIG 4 Survival of WT RS218 and RS218 $\Delta nlpI$ in NHS where the classical, lectin, or alternative complement pathway was blocked. (A) Survival of *E. coli* strains in serum in which the classical complement pathway was inhibited by an antibody against C1q (10); (B) survival of the bacterial strains in serum in which the alternative complement pathway was inhibited by bentonite (13); (C) survival of the bacterial strains in serum in which the lectin complement pathway was inhibited by mannose (25). The survival rates were calculated by the ratio of the bacterial counts to those of the original inocula at different time points. The data are representative of three independent experiments performed in triplicate and are presented as the mean $\pm$ SD. Anti-C1q-NHS, anti-C1q antibody-treated NHS; HI-Anti-C1q-NHS, anti-C1q antibody-treated HI-NHS; Ben-NHS, bentonite-treated NHS; HI-Ben-NHS, bentonite-treated HI-NHS; Man-NHS, mannose-treated NHS; and HI-Man-NHS, mannose-treated HI-NHS.

The NlpI protein may not directly interact with C4bp, although NlpI is exposed on the bacterial surface. Given that NlpI has been shown to localize at the outer membrane fraction of *E. coli* (35), it is reasonable to speculate that NlpI may directly interact with and recruit C4bp to *E. coli*; i.e., the exposure of NlpI to the bacterial surface is a prerequisite for the potential NlpI-C4bp interaction. As shown in Fig. 7A, the C-terminally His₆-tagged NlpI was expressed (using WT RS218 harboring plasmid pN110-His, which encodes this recombinant protein) and could be detected on the surface of *E. coli* by immunomicroscopy with an anti-His₆ antibody. In addition, transcomplementation of RS218 $\Delta nlpI$ with pN110-His restored the mutant's serum survival to the level of WT RS218, indicating that the C-terminally His₆-tagged NlpI is functional in *E. coli* (Fig. 7B).

Next, we investigated whether C4bp directly interacts with NlpI on the surface of NMEC using a purified NlpI recombinant protein for blocking assays. The purified C-terminally His₆-tagged NlpI, however, was not stable in the experiment solution; the protein precipitated readily, and it was not suitable for the assays. Therefore, we used an N-terminally His₆-tagged NlpI, which was soluble in the experiment solution. We found that the purified recombinant NlpI did not block C4bp deposition onto the surface of WT RS218 when the bacteria were incubated with the purified C4bp protein (see Fig. S4 in the supplemental material). Additionally, the addition of the N-terminally His₆-tagged NlpI protein did not affect the survival of WT RS218 in NHS (see Fig. S5 in the supplemental material). In agreement with these findings, we

found that preincubation of WT RS218 with heat-inactivated rabbit NlpI antiserum (see the Materials and Methods section and Fig. S2 in the supplemental material) did not affect the bacterium's ability to recruit the purified C4bp protein (see Fig. S6 in the supplemental material). These results suggest that NlpI may not directly interact with C4bp in NHS, although NlpI is exposed on the bacterial surface.

The involvement of NlpI in C4bp deposition on the surface of NMEC may act through OmpA. The components of the bacterial envelope, including the K1 capsule, lipopolysaccharide (LPS), and the outer membrane protein OmpA, have been shown to contribute to the resistance of *E. coli* to complement-mediated serum killing (20, 27, 28). We found that the levels of K1 capsule and LPS on WT RS218 and RS218 $\Delta nlpI$ were similar (data not shown). Our previous study also showed that deletion of *nlpI* does not affect the expression of OmpA on the surface of *E. coli* (35). These findings exclude the possibility that the involvement of NlpI in *E. coli* resistance to serum killing occurs through affecting the expression levels of these bacterial components.

OmpA has been shown to be involved in the deposition of C4bp on the surface of NMEC. This protein directly interacts with C4bp on the bacterial surface, allowing bacterial evasion of serum killing mediated by the classical complement pathway (28). Thus, we investigated how OmpA and NlpI contribute to C4bp deposition on NMEC. The levels of C4bp deposition on WT RS218, RS218 $\Delta nlpI$, RS218 $\Delta ompA$ (the *ompA* mutant of RS218), and RS218 $\Delta nlpI \Delta ompA$ (the *nlpI ompA* double mutant of RS218)

FIG 5 Deposition of C4bp on WT RS218 and RS218 $\Delta nlpI$ after incubation in NHS. (A) The levels of C4bp deposition on RS218 $\Delta nlpI$ determined by flow cytometry were compared with those on WT RS218. The data are presented as the relative mean fluorescent intensity (MFI) compared to the deposition on WT RS218. These quantitative data were derived from three independent experiments. *, $P < 0.05$. (B) Flow cytometry histogram of C4bp deposition on the bacteria after 60 min of incubation in NHS. (C) Deposition of purified C4bp on the bacteria. Bacteria (2×10^7 CFU) were incubated with 5 μ g of purified C4bp in 500 μ l of Veronal buffer for 60 min. The bacterial lysates were subjected to Western blot analysis to determine the level of C4bp deposition in each sample. The OmpA levels were determined by Western blot analysis and served as a loading control for the bacteria.

were measured after incubation with NHS for 60 min. All the mutants exhibited similar levels of C4bp deposition, but their levels of C4bp deposition were significantly decreased compared to those of WT RS218 (Fig. 8). These findings suggest that NlpI and OmpA may work dependently to recruit C4bp to the surface of *E.*

coli. Furthermore, the CCP3 domain of C4bp has been shown to be responsible for its interaction with OmpA (28). The peptide CCP3-4 (ENETIGVWRP), whose sequence is derived from the CCP3 domain, is able to inhibit the OmpA-C4bp interaction by competitively binding to OmpA and thus increase the serum killing of NMEC (28). We found that the serum survival of RS218 $\Delta nlpI$ was not affected by adding CCP3-3 to hinder the C4bp-OmpA interaction (Fig. 9), which suggests that the OmpA on RS218 $\Delta nlpI$ is not functional in protecting bacteria from complement attack. These findings collectively suggest that the involvement of NlpI in C4bp deposition on NMEC may be by affecting the function of OmpA.

DISCUSSION

Most cases of *E. coli* meningitis develop as a consequence of BBB traversal by the bacteria circulating in the bloodstream. To accomplish such penetration of the BBB, two major pathogenesis steps are required for the pathogen, i.e., induction of a high-level bacteremia and binding to and invasion of HBMECs (14, 15). We have previously shown that NlpI is involved in the binding to and invasion of HBMECs by NMEC (35). In the present study, we examined the role of NlpI in bacteremia and evasion of the host innate defense. Our results demonstrated new lines of evidence indicating that NlpI contributed to high-level NMEC bacteremia and was involved in NMEC evasion of serum killing mediated by the complement system, the first line of innate immunity against invading pathogens. NlpI was found to be mainly responsible for avoiding the activation of the classical complement cascade downstream of C1q binding, the initial step of the classical activation. The mechanism by which NlpI avoided activation of the classical complement system was most likely through deposition of C4bp, which is a complement inhibitor of the classical and lectin pathways. NlpI may be functionally required for the interaction be-

FIG 6 Survival of RS218 $\Delta nlpI$ compared with that of WT RS218 in C4bp-depleted NHS after 120 min of incubation. The results are presented as relative survival rates compared to the survival rates of WT RS218 in the control NHS. The data shown are representative of three independent experiments performed in triplicate and are presented as the mean $\pm$ SD. C4bp-depleted NHS, NHS preabsorbed with beads that were coated with goat anti-C4bp IgG; control NHS, NHS preabsorbed with beads that were not coated with antibodies; +, supplementation with purified C4bp protein; -, no C4bp supplementation; *, $P < 0.05$; **, $P < 0.01$.

FIG 7 Surface exposure of NlpI on NMEC RS218. (A) WT RS218 harboring pNI10-His was investigated by immunofluorescence microscopy using anti-His₆ antibody (right) and by light microscopy (left); (B) WT RS218 harboring the control plasmid, pTrc99A, was investigated by immunofluorescence microscopy with anti-His₆ antibody (right) and by light microscopy (left); (C) survival of RS218 $\Delta nlpI$ harboring pNI10-His or pTrc99A was compared with that of WT RS218 harboring pTrc99A after incubation in NHS for 120 min. The results are presented as the relative survival rates compared to the survival rate of WT RS218 harboring pTrc99A. The results are representative of three independent experiments performed in triplicate and are presented as the mean $\pm$ SD. **, $P < 0.01$.

tween C4bp and OmpA, which is an outer membrane protein that is shown to directly interact with C4bp on the bacterial surface (28).

Our finding supports the suggestion that NlpI is mainly involved in NMEC evasion of the attack by the classical complement pathway (Fig. 4), but the possibility of a role of NlpI in avoiding serum killing mediated by the lectin and alternative complement pathways cannot be excluded. C4bp is also a suppressor of the lectin pathway. To initiate the lectin pathway, the serum MBL (also named mannose-binding protein [MBP]) is required to bind to the surfaces of invading microorganisms in the bloodstream (26). It is known that the serum concentration of MBL is age dependent; the concentration reaches its highest level in newborns less than 1 week old and declines with advancing age (36, 37). Therefore, the lectin pathway may play a more important role against invading pathogens in neonates than in adults. Accord-

ingly, the NlpI-dependent C4bp deposition on NMEC may have a more prominent role in protecting the bacteria from killing mediated by the lectin pathway in neonates than in adults. The alternative pathway is initiated through spontaneous hydrolysis of the serum protein C3 to produce C3b, which in turn interacts with the alternative pathway components factors B and F, leading to the activation of the downstream alternative cascade. However, the C3b generated by the classical and lectin pathways can also interact with these alternative pathway components, leading to the same downstream activation (39). Thus, the contribution of NlpI to NMEC in avoiding the activation of the classical pathway may indirectly decrease the activation of the alternative pathway in the bloodstream.

In addition to the involvement of C4bp recruitment, NlpI may have an alternative function to evade complement attack. For example, WT RS218 exhibited a higher level of survival than RS218 $\Delta nlpI$ in C4bp-depleted serum, although C4bp depletion de-

FIG 8 Comparison of C4bp deposition on WT RS218, RS218 $\Delta nlpI$, RS218 $\Delta ompA$, and RS218 $\Delta nlpI \Delta ompA$ after incubation in NHS. The levels of C4bp deposition on bacterial cells were determined by flow cytometry after the cells were incubated in NHS for 60 min. The data are presented as the relative mean fluorescent intensity (MFI) compared to that of WT RS218. These data were derived from three independent experiments. *, $P < 0.05$.

FIG 9 Effects of treatment with CCP3-4 peptide on serum survival of WT RS218 and RS218 $\Delta nlpI$. *E. coli* cells were preincubated with 100 μ M CCP3-4 for 60 min at 37°C and then subjected to serum survival assays. The results are presented as the relative survival rates compared to the survival rate of WT RS218 without CCP3-4 treatment. The results are representative of three independent experiments performed in triplicate and are presented as the mean $\pm$ SD. *, $P < 0.05$.

creased the survival of WT RS218 in serum (Fig. 6). These results are less likely due to the function of the residual C4bp in the C4bp-depleted serum because most of the C4bp proteins were found to be removed in this serum (see Fig. S1 in the supplemental material). Therefore, these results suggest that the decreased ability of RS218 $\Delta nlpI$ to recruit C4bp may not be fully responsible for the decreased ability of the mutant to survive in serum. Accordingly, NlpI may be involved in other mechanisms that facilitate bacterial evasion of complement attack.

Two lines of our results suggest that NlpI and OmpA may work dependently to recruit C4bp on NMEC. First, the double-knock-out mutant (RS218 $\Delta nlpI \Delta ompA$) and single-knockout mutants (RS218 $\Delta nlpI$ and RS218 $\Delta ompA$) exhibited a similar level of decreased ability in recruiting C4bp compared to WT RS218. Second, the CCP3-4 peptide, which is shown to be able to specifically block the interaction between OmpA and C4bp to decrease the serum survival of NMEC (28), did not affect the serum survival of RS218 $\Delta nlpI$, suggesting that OmpA may not be functional to interact with C4bp on *E. coli* with a deletion of *nlpI*. Consistently, our studies have previously shown that the *nlpI* mutant and the *ompA* mutant of NMEC are similarly defective in interaction with host cells (3, 20, 35). It is known that NMEC can trigger the activation of the host signaling molecule cytosolic phospholipase A2 (cPLA₂ α) of HBMECs to facilitate the invasion of the host cells (3). We have shown that RS218 $\Delta nlpI$ exhibits a decreased ability in triggering the activation of cPLA₂ α of HBMECs compared with WT RS218 (35). Recently, Maruvada and Kim have demonstrated that OmpA also contributes to cPLA₂ α activation during NMEC RS218 invasion of HBMECs and that two of the four surface-exposed loops (loops 1 and 2) of this outer membrane protein are responsible for triggering this activation (20). Loops 1 and 2 of OmpA are also shown to interact with C4bp and thus facilitate the survival of NMEC in serum (20, 28). Collectively, it is likely that NlpI is required for allowing loops 1 and 2 of OmpA to interact with the complement regulator for serum survival and the host receptor(s) to trigger cPLA₂ α activation in HBMEC invasion. Thus, NlpI may be involved in the proper conformation of the OmpA surface-exposed loops to interact with C4bp on the bacterial surface. It has been shown that *E. coli* strains with an *nlpI* deletion overproduce outer membrane vesicles (21), which implies that deletion of this outer membrane protein may alter the properties of the bacterial outer membrane. Such alteration may hinder the interaction between OmpA and C4bp by hampering the access of C4bp to the exposed OmpA loops or by interfering with the structure of OmpA on the outer membrane. Nevertheless, the possibility that NlpI directly interacts with C4bp to facilitate the OmpA-mediated deposition of C4bp cannot be excluded, because there might be a possibility that the NlpI recombinant protein that we used may not have the proper conformation to interact with C4bp and that the epitopes of NlpI recognized by the antiserum may not be the critical part that interacts with C4bp. Additional studies are required to examine whether NlpI can directly interact with C4bp to facilitate deposition of this regulator.

NlpI contains five tetratricopeptide repeat (TPR) motifs (24, 32, 41), which mediate intermolecular protein-protein interactions that lead to the formation of protein complexes (4, 17, 30). The NlpI protein has been proposed to form homodimers on the bacterial membrane, and a cleft that is located at the interface of this homodimer has been predicted to be a ligand-binding site, although the ligand has not been identified. The interaction of the

NlpI dimers on the outer membrane with this unidentified ligand may be required for the C4bp-binding function on the bacteria. A more detailed study is necessary to identify the interacting NlpI ligand.

In conclusion, our study demonstrates a new role for NlpI in *E. coli* bacteremia and evasion of the classical complement host defense pathway. We stressed that NlpI is required for NMEC to cause high-level bacteremia and cross the BBB, which are two pathogenic steps critical for NMEC to cause meningitis (14, 15). Because NlpI is common in *E. coli* isolates from blood and CSF (43), the functional importance of NlpI identified in this study warrants further investigation of this protein as a novel target for prevention and therapy of invasive *E. coli* infection.

ACKNOWLEDGMENTS

We thank Akiko Okuda-Tadokoro for kindly providing plasmids pNII and pCL1920.

This work was supported by the National Science Council, Taiwan (grant no. NSC99-2320-B-006-005-MY3), and the Multidisciplinary Center of Excellence for Clinical Trial and Research, Department of Health, Executive Yuan, Taiwan (grant no. DOH100-TD-B-111-002).

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