Nucleotide receptors mediate protection against neonatal sepsis and meningitis caused by alpha-hemolysin expressing *Escherichia coli* K1

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**Abstract**

Neonatal meningitis-associated *Escherichia coli* (NMEC) is among the leading causes of bacterial meningitis and sepsis in newborn infants. Several virulence factors have been identified as common among NMEC, and have been shown to play an important role in the development of bacteremia and/or meningitis. However, there is significant variability in virulence factor expression between NMEC isolates, and relatively little research has been done to assess the impact of variable virulence factor expression on immune cell activation and the outcome of infection. Here, we investigated the role of NMEC strain-dependent P2X receptor (P2XR) signaling on the outcome of infection in neonatal mice. We found that alpha-hemolysin (HlyA)-expressing NMEC (HlyA+) induced robust P2XR-dependent macrophage cell death in vitro, while HlyA− NMEC did not. P2XR-dependent cell death was inflammasome independent, suggesting an uncoupling of P2XR and inflammasome activation in the context of NMEC infection. In vivo inhibition of P2XR was associated with increased mortality in neonatal mice infected with HlyA+ NMEC, but had no effect on the survival of neonatal mice infected with HlyA− NMEC. Furthermore, we found that P2XR-dependent protection against HlyA+ NMEC in vivo required macrophages, but not neutrophils or NLRP3. Taken together, these data suggest that HlyA+ NMEC activates P2XRs which in turn confers macrophage-dependent protection against infection in neonates. In addition, our findings indicate that strain-dependent virulence factor expression should be taken into account when studying the immune response to NMEC.

**Abbreviations**: BBB, blood-brain barrier; BBG, brilliant blue G; CM, complete media; CNF-1, cytotoxic necrotizing factor-1; ExPEC, extraintestinal pathogenic *Escherichia coli*; GSDMD, gasdermin-D; HlyA, α-hemolysin; i.p., intraperitoneal; Lac, lactose; LDH, lactate dehydrogenase; MOI, multiplicity of infection; NLRP3, NLR family pyrin domain containing 3, P2R, purinergic receptor; NMEC, neonatal meningitis-associated *Escherichia coli*; TLR, toll-like receptor; UPEC, uropathogenic *Escherichia coli*.

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1 | INTRODUCTION

*Escherichia coli* is a leading cause of sepsis in newborn infants, and the leading cause of meningitis in premature infants.\(^1\,^2\) Neonatal meningitis-associated *E. coli* (NMEC) is a distinct pathotype of extraintestinal pathogenic *E. coli* (ExPEC) associated with both sepsis and meningitis in infants.\(^3\,^4\) While appropriate antibiotic therapy has significantly improved clinical outcomes, several limitations still exist. Antibiotic treatment during severe bacteremia can cause significant release of endotoxin leading to septic shock and organ failure.\(^5\) Furthermore, a number of antibiotic-resistant NMEC strains have been recently identified which may complicate treatment.\(^6\,^12\)

Finally, even when antibiotic treatment is successful, infants that survive these infections are at an increased risk of life-long neurologic sequelae ranging from learning disabilities to seizure disorders.\(^1,^13\) These continued limitations precipitate the need for new therapeutics to improve clinical outcomes and overcome the rise in antibiotic resistance.

While NMEC shares many similarities, including a propensity to express the K1 capsule antigen, there is a high degree of variability in virulence factor expression.\(^3\,^4\) To that end, we investigated differences in immune stimulation by, and virulence of, two strains of NMEC. Both NMEC O18 and NMEC RS218 were originally cultured from cerebrospinal fluid of infants with meningitis.\(^14,^15\) NMEC O18 and NMEC RS218 have been fully sequenced, and both are O18:H7:K1 *E. coli*.\(^14,^15\) One important difference is that NMEC RS218 is known to express the toxins cytotoxic necrotizing factor-1 (CNF-1) and α-hemolysin (HlyA), while NMEC O18 does not. CNF-1 is a toxin that activates Rho-family GTP-binding proteins to regulate the actin cytoskeleton of cells, and can subsequently induce numerous effects, including increasing nonspecific endocytosis and micropinocytosis.\(^16,^17\) CNF-1 has also been shown to aid NMEC in crossing the blood-brain barrier.\(^18\)

HlyA is a pore-forming toxin harbored by approximately 30% of NMEC isolates,\(^3\) and has also been associated with increased severity of uropathogenic *E. coli* (UPEC) infection.\(^19,^20\) HlyA interacts with several immune pathways, including the NLRP3 inflammasome and purinergic receptor pathways, in the context of UPEC infection.\(^21\,^25\) While these interactions could contribute to increased pathogenicity of HlyA+ bacteria, under certain conditions they may also induce protective immune responses that improve bacterial clearance. To our knowledge, the role of HlyA in NMEC infection has not been studied.

The NLRP3 inflammasome is thought to be activated in response to cellular damage and subsequent K\(^+\) efflux.\(^26\) Therefore, it is not surprising that HlyA has been associated with increased NLRP3 activation.\(^22,^23\) Following activation, NLRP3, like other inflammasomes, activates caspase-1, which in turn cleaves pro-IL-1β to its active form.\(^27\) Activated caspase-1 also cleaves gasdermin-D (GSDMD) allowing the N-terminal segment to polymerize in the cell membrane forming a pore through which IL-1β and other cytokines can escape the cell.\(^28\) Accumulation of GSDMD pores in the cell membrane can also lead to the lytic form of cell death known as pyroptosis.\(^28\)

Cell membrane purinergic receptors (P2Rs) bind to nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate, which are released into the extracellular space under both physiologic and pathologic conditions.\(^29,^30\) P2Rs can be subdivided into two main types (P2XR and P2YR) based on the receptor type.\(^29\) P2XRs are membrane-bound, cation-selective, ligand-gated ion channels that are activated primarily by extracellular ATP.\(^30\) Large quantities of ATP are released into the extracellular space following cell damage, such as from HlyA pores.\(^29\) While the activation of P2XRs can be associated with overzealous inflammation and detrimental effects on infection outcome, P2XRs are also involved in chemotaxis and activation of many immune cells and can play an important role in developing a protective immune response.\(^29\) We, therefore, assessed how differential activation of P2XRs by NMEC impacts the outcome of infection in neonatal mice.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

All experiments were performed in biosafety level 2 facilities at the University of Missouri. *Escherichia coli* strains were provided by Dr. Lisa Nolan and Dr. Catherine Logue at the University of Georgia. *E. coli* was grown from frozen stocks on MacConkey agar (BD) plates at 37°C and then cultured in Luria-Bertani broth (BD) for approximately 18 h at 37°C with constant shaking. Bacteria were washed...
and resuspended in phosphate-buffered saline (PBS) for dilution to final working concentrations. Starting concentrations were approximated by measuring turbidity with a spectrophotometer at OD600 and were confirmed by enumerating viable bacteria from serial dilutions plated on MacConkey agar.

The E. coli strains used in this study were isolated from neonatal meningitis cases in the US and the Netherlands and have been described elsewhere. A description of the strains can be found in Table 1. Analysis of phylogenetic groups was performed as previously described and O serogrouping was performed at the E. coli Reference Center, Pennsylvania State University. To identify lactose-fermenting (Lac+) strains, bacteria were plated on MacConkey agar with incubation at 37°C. Hemolytic ability was assessed by plating test strains on blood agar at 37°C for 24–48 h and observation for zones of hemolysis. NMEC strains were genotyped for cnf1 and hlyA using primers described by Wijetunge et al. Expression of nlp1 was used as a positive control and was identified in all strains.

2.2 | Mice

All animal work was performed in accordance with University of Missouri Institutional Care and Use Committee policies. Mice were housed in University of Missouri vivaria with a 12-h light/dark cycle and ad libitum access to rodent chow and water. Experiments were conducted using age- and sex-matched mice on a C57BL/6J background. C57BL/6J (wildtype), NLRP3−/−, and Caspase 1/11−/− (Casp1/11−/−) breeder pairs were originally obtained from Jackson Laboratory. Casp1/11−/− were crossed with mice lacking caspase-8 in myeloid cells (Caspase8−/−/LysMcre) to generate Caspase1/11−/−/Caspase8−/−/LysMcre mice. Caspase8−/−/LysMcre mice were generated initially by crossing Caspase8−/− mice and Lyz2M-cre expressing mice (both from Jackson Laboratory). P2X7−/− mice were provided by Dr. Gary Weisman at the University of Missouri.

2.3 | Macrophage generation

Macrophages were elicited by administering thioglycolate (BD) intraperitoneally (i.p.) to 6–12-week-old mice. Mice were euthanized 3–4 days post-thioglycolate treatment and the peritoneum was washed with 10-ml RPMI 1640 (Thermo Fisher). Cells were washed, resuspended in complete media [CM; RPMI 1640, 10 mM HEPES buffer, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, (all from Thermo Fisher), and 10% heat-inactivated fetal bovine serum (FBS; Millipore Sigma)], plated at 1 x 10^6 cells/ml and incubated at 37°C with 5% CO₂ overnight to allow for adhesion to the plate prior to use in experiments.

| Strain     | Phylogroup | O type | Lac | Hemolysis | CNF-1 | HlyA |
|------------|------------|--------|-----|-----------|-------|------|
| NMEC RS218 | B2         | 18     | +   | β         | +     | +    |
| NMEC 6     | B2         | 6      | +   | β         | +     | +    |
| NMEC 12    | B2         | 6      | +   | β         | +     | +    |
| NMEC 26    | B2         | 21     | −   | β         | −     | +    |
| NMEC 45    | A          | 23     | +   | β         | +     | +    |
| NMEC 81    | B2         | 12     | −   | β         | −     | +    |
| NMEC 87 (C5)| B2        | 18     | +   | β         | +     | +    |
| NMEC 42    | B2         | auto   | +   | γ         | +     | +    |
| NMEC O18   | B2         | 18     | +   | γ         | −     | −    |
| NMEC 4     | B2         | 18     | +   | γ         | −     | −    |
| NMEC 15    | B2         | 18     | +   | γ         | −     | −    |
| NMEC 20    | A          | 7      | +   | γ         | −     | −    |
| NMEC 71    | B2         | 21     | +   | γ         | −     | −    |
| NMEC 82    | B2         | 1      | +   | γ         | −     | −    |
| NMEC 83    | D          | 1      | +   | γ         | −     | −    |

Note: NMEC strains used in this study. Strains were characterized as described in Materials and Methods. Strains were determined to be β-hemolytic if a clear region formed around colonies, and γ-hemolytic if no hemolysis was appreciated. Auto=autoagglutination.
2.4 | In vitro infection, cytokine quantification and assessment of cell death

Cells were washed with PBS and either CM or Opti-MEM (Thermo Fisher) was added prior to infection with *E. coli*. Treatment with cycloheximide (10 μM, MP Biomedicals), potassium chloride (KCl; 50 mM, Sigma), glycine (10 mM, Tocris), necrosis-releasing agent (20 μM, Tocris), necrostatin-1 (10 μM, Sigma), z-VAD (100 μM, Tonbo), Brilliant Blue-G (BBG; 100 μM, Sigma), BB-FCF (Sigma 100 μM), Bay-1797 (10 μM; MedChemExpress), apyrase (10 U/ml, Sigma), hexokinase (20 U/ml in 10 mM glucose), or QX-314 (Sigma, 5 mM) was initiated at the time cells were washed, approximately 1 h prior to infection. Cells were infected with *E. coli* at a multiplicity of infection (MOI) of 0.1, 1, 10, or 100 as described in the figure legends. Supernatants were collected 5 h post-infection, treated with penicillin-streptomycin, and stored at −20°C until the time of analysis. For LPS/nigericin groups, 200 ng/ml LPS (Sigma, serotype O:111, B4) was added to wells and 20-μM nigericin (AdipoGen) was added 4 h post-LPS treatment. Supernatants were collected 1 h following nigericin treatment and frozen until the time of analysis. Cell supernatants were assessed for cytokine production using ELISA kits: Mouse IL-1β (Invitrogen), and Mouse IL-6 (eBioscience) according to the manufacturer’s instructions.

For the measurement of cell death, cells were cultured in Opti-MEM and infected as described above. Cytotoxicity was determined by assaying supernatants for lactate dehydrogenase (LDH) release adhering to the manufacturer’s protocol (Roche, Cytotoxicity Detection Kit plus).

To assess the uptake of bacteria and bacterial killing, macrophages were infected with NMEC RS218 or NMEC O18. One-hour post-infection, cells were washed with sterile PBS and media was replaced with CM containing 50 μg/ml gentamicin (Fresenius Kabi). Macrophages were then washed and lysed with sterile H2O 1.5-, 5-, or 24-h post-infection. Lysates were plated on MacConkey agar to determine intracellular bacterial loads.

2.5 | Quantitative reverse transcriptase PCR

Cells were infected as described above. At designated time points, RNA was isolated from cell lysates using the Qiagen RNeasy kit. cDNA was generated using the Superscript III First-Strand Synthesis System with oligo dT primers. The quantitative reverse transcriptase PCR was set up in duplicate and data were collected on an Applied Biosystems StepOne Real-Time PCR System. Relative IL-1β mRNA in relation to GAPDH was quantified by measuring SYBR green (Thermo Fisher) incorporation. The following primers from Integrated DNA Technologies were used: IL-1β forward, 5′-ATCCACCTTTTGACAGTGAT-3′ and reverse, 5′-ATCCAGACGCGCTCAAA-3′; GAPDH forward, 5′-GTGGACCTGGCTCCTAT-3′ and reverse, 5′-GGGTGACGAACCTTATTG-3′.

2.6 | Neonatal mouse infection

Three-day-old mice were infected i.p. with 50–10 000 CFU of NMEC RS218 or NMEC O18 as described in the figure legends. Experiments were performed with intra-litter control animals to limit the role of litter-to-litter variability in our results. Male and female mice were split approximately evenly between treatment groups within litters. Data from multiple litters were pooled and n values for each graph are included in the figure legends. BBG (Sigma, 50 mg/kg sterile-filtered in 25 μl PBS) or an equivalent volume of PBS were delivered i.p. approximately 2 h prior to infection and readministered 24 h later for survival studies. Apyrase was given i.p. at a dose of 15 U/mouse in PBS 30 minutes prior to infection and readministered 16 h later for survival studies (control animals received PBS).

To assess survival, mice were monitored at 2–4 h intervals starting 16–18 h post-infection. During these experiments, blood was collected 18 h post-infection via the facial vein for confirmation of bacteremia. Mice that appeared unresponsive during monitoring were euthanized using an AVMA approved method and recorded as mortality.

For assessment of bacterial loads and cytokine production at 6-, 12-, or 18-h post-infection, mice were anesthetized with isoflurane and euthanized by cardiac puncture and exsanguination. The peritoneum was washed with 200 μl sterile PBS. The brain and spleen were then removed and homogenized in 500 μl sterile PBS. Serial dilutions of the blood, peritoneal lavage fluid, brain, and spleen were plated on MacConkey agar to determine bacterial loads. Peritoneal lavage samples were then centrifuged to remove host and bacterial cells, and all samples were treated with antibiotics and frozen at −20°C for future use in the assessment of cytokine production. Cytokine production was assessed using a multiplexed magnetic bead assay (eBioscience) and the Luminex MagPix system according to the manufacturer’s instructions (MilliporeSigma).

2.7 | Neutrophil and macrophage depletions

To deplete neutrophils, mice were treated with 15 μg (approximately 5 mg/kg) of anti-Ly6G antibody (clone
in the figure legends. All error bars represent the standard deviation of the sample mean.

2.8 Statistical analysis

All statistical analysis was performed in SigmaPlot 14.0. In vivo CFU results were analyzed by Mann-Whitney Rank Sum analysis with significance set at \( p < .05 \). Statistical significance of survival studies was determined using log-rank analysis with significance set at \( p < .05 \). In vitro data were normally distributed, and, therefore, analyzed via the Student’s \( t \)-test (when comparing two groups) or via ANOVA followed by Tukey’s test (when comparing 3 or more groups) with significance set at \( p < .05 \) as described in the figure legends. All error bars represent the standard deviation of the sample mean.

3 RESULTS

3.1 NMEC O18 and NMEC R218 display differential virulence and macrophage activation

It has previously been shown that NMEC O18 is significantly more virulent than NMEC RS218 in a neonatal rat model of infection.\(^{49}\) To determine if NMEC O18 was also more virulent in a neonatal mouse model, we infected 3-day-old C57BL/6 mice with \( \sim 10^5 \) NMEC O18 or NMEC RS218 i.p. We found that mice infected with NMEC O18 had \( \sim 100 \) 000-fold higher bacterial loads in blood, peritoneal lavage, and brain 18 h post-infection than mice infected with NMEC RS218, confirming that NMEC O18 is significantly more virulent (Figure 1A–C).

It is surprising that NMEC RS218, which harbors both HlyA and CNF-1, is less virulent than the non-toxin-producing NMEC O18, as both these toxins are associated with increased disease severity during UPEC infection.\(^{19,20,50}\) We hypothesized that HlyA and/or CNF-1 may trigger protective innate immune pathways which contribute to the enhanced bacterial clearance of NMEC RS218 compared to NMEC O18. CNF-1 has been shown to facilitate NMEC crossing of the BBB; however, it is also known to increase nonspecific endocytosis of material by macrophages.\(^{16}\) To determine the potential role of CNF-1 in NMEC RS218 infection, we assessed bacterial uptake and killing by macrophages. We found that macrophage uptake of NMEC RS218 was higher than that of NMEC O18 (Figure S2A). However, there was no apparent difference between strains in the survival of bacteria that were phagocytosed (Figure S2B).

When we assessed secretion of IL-6 by macrophages infected with either NMEC O18 or NMEC RS218, we found that macrophages infected with either bacteria produced similar levels of IL-6 (Figure 1D). We next measured secretion of IL-1\( \beta \) and found that NMEC O18 infected macrophages secreted significantly less IL-1\( \beta \) compared to NMEC RS218 infected cells (Figure 1E). IL-1\( \beta \) requires a two-step process for activation and secretion. In the first step, IL-1\( \beta \) mRNA is induced following TLR activation, and pro-IL-1\( \beta \) is produced. The second step requires activation of inflammasomes, which activate caspase-1 to cleave pro-IL-1\( \beta \) to its active form. Once cleaved, IL-1\( \beta \) can then be secreted from cell.\(^{27}\) We found that both NMEC O18 and NMEC RS218 induced a similar amount of IL-1\( \beta \) mRNA (Figure 1F), indicating that a difference in inflammasome activation, rather than transcription, results in differential secretion of IL-1\( \beta \) by NMEC RS218 and NMEC O18. HlyA is known to activate the NLRP3 inflammasome, and, therefore, NLRP3 activation could serve as a differential elicited immune response, that might explain the decreased virulence of NMEC RS218 compared to NMEC O18. However, we previously found that IL-1 signaling did not play a significant role in the protection of wildtype neonatal mice against NMEC RS218 infection,\(^{51}\) indicating a need to search for other differential responses between the two bacterial strains.

In addition to IL-1\( \beta \) secretion, inflammasome activation also leads to cleavage of GSDMD. Following cleavage, the N-terminal fragment of GSDMD polymerizes within the cell membrane to create pores through which cytokines, particularly IL-1\( \beta \) and IL-18 are able to leave the cell.\(^{28}\) Furthermore, these pores can ultimately lead to destabilization of the cell membrane, resulting in the lytic form of cell death known as pyroptosis.\(^{52}\) To determine if NMEC O18 and NMEC RS218 induced differential cell death responses, LDH was measured in supernatants from macrophages infected with both bacteria. We found that while NMEC RS218 induced robust cell death, NMEC O18 did not (Figure 1G).
To determine whether macrophage cell death and IL-1β secretion in response to NMEC RS218 infection required the bacteria to be taken up by the cell, we treated macrophages with cytochalasin-D, a suppressor of phagocytosis, prior to infection with NMEC RS218. We found that cytochalasin-D led to significantly reduced intracellular bacteria (Figure S2C). Secretions of IL-1β and IL-6 were also significantly decreased, but not completely abrogated in cytochalasin-D treated macrophages (Figure S2D,E), indicating that secretion of these cytokines is at least partially dependent on internalization of bacteria by the macrophages. Interestingly, we did not see a significant difference in cell death between control and cytochalasin-D-treated cells (Figure S2F), indicating that extracellular stimuli are sufficient to induce cell death following NMEC RS218 infection, and suggesting that there may be different pathways involved in cytokine secretion versus cell death during NMEC RS218 infection.

To further investigate cell death in response to NMEC RS218, we pre-treated macrophages with either glycine, which is known to stabilize cell membranes and limit cell lysis, necrosulfonamide, which inhibits GSDMD from polymerizing in the cell membrane, or KCl, which is known to block NLRP3 activation by limiting potassium efflux. Combined treatment with LPS and nigericin was used as a control for NLRP3/GSDMD dependent IL-1β secretion and cell death. As expected, the addition of glycine had no effect on IL-1β secretion in response to LPS/nigericin treatment or NMEC RS218 infection but markedly reduced cell death (Figure 2A,B). Treatment with either necrosulfonamide or KCl abrogated secretion of IL-1β in response to both LPS/nigericin and NMEC RS218 infection (Figure 2B). Interestingly, while necrosulfonamide and KCl significantly inhibited cell death following LPS/nigericin treatment, neither NLRP3 nor GSDMD inhibition had a significant effect on cell death following NMEC RS218 infection (Figure 2B). To further confirm that cell death was independent of NLRP3 inflammasome activation, macrophages from wildtype, NLRP3−/− and Caspase 1/11−/− mice were infected.
with NMEC RS218 and we found that inflammasome deficiency did not reduce cell death (Figure 2C), further indicating that macrophage cell death in response to NMEC RS218 infection is not mediated by NLRP3 or caspases 1 and 11. It has been reported that in cells lacking caspase-1 and 11, caspase-8 serves a compensatory role to mediate cell death.53 Therefore, we infected macrophages lacking caspases-1, -11, and -8, and found that NMEC RS218 induced cell death was not altered by a combined deficiency of caspases 1, 8, and 11 (Figure S3A). Furthermore, we treated wildtype cells with necrostatin-1 to inhibit the necroptotic cell death pathway and did not see a significant effect of inhibition on cell death (Figure S3B). Finally, it is known that pyroptosis, necroptosis, and apoptosis are highly interconnected and frequently compensatory.54 We, therefore, assessed whether blocking all three pathways simultaneously would have a significant impact on cell death. By infecting macrophages lacking caspases 1, 8, and 11 in conjunction with treatment with the pan-caspase inhibitor, Z-VAD, and necrostatin-1, we determined that inhibition of all three pathways had no effect on cell death (Figure S3C). Collectively, these results suggest that the cell death induced by NMEC RS218 is more likely due to non-programmed necrosis than caspase-dependent cell death.

3.2 | P2XRs mediate macrophage cell death, but not IL-1β secretion, in response to NMEC RS218

The HlyA pore can elicit the release of ATP from host cells into the extracellular space which can result in activation of purinergic receptors that can lead to cell death via caspase-dependent and -independent mechanisms,55–58

As we found that NMEC RS218-induced cell death is caspase-independent (Figure 2 and Figure S3), we assessed the role of purinergic receptors in vitro by pre-treating macrophages with Brilliant Blue G (BBG), which is most specific for P2X7, but can inhibit other P2X receptors at higher concentrations.59 Interestingly, we found that BBG-treatment significantly decreased cell death, but did not have a significant impact on IL-1β secretion (Figure 3A,B), corroborating our previous results (Figure 2) indicating uncoupling of NLRP3-dependent IL-1β production from the cell death response. To verify that NMEC RS218-induced cell death was due to the sensing of ATP, we pre-treated macrophages with apyrase, which hydrolyzes ATP, or hexokinase, an ATP scavenger.44 Similar to what we observed with BBG, both apyrase and hexokinase inhibited cell death without affecting IL-1β production (Figure 3C,D) which confirms that ATP release elicited by NMEC RS218 results in host cell death.

It has previously been suggested that HlyA interacts with the pannexin-1 receptor to release ATP from the cell.44 To determine the role of pannexin-1 in purinergic receptor activation and macrophage cell death in response to NMEC RS218, we pre-treated cells with the pannexin-1 inhibitor BB-FCF.62 We found that in contrast to BBG, BB-FCF did not significantly impact IL-1β secretion or cell death following NMEC RS218 infection (Figure S4A,B), suggesting that NMEC RS218 is able to induce the release of ATP and purinergic receptor activation in the absence of pannexin-1 signaling.

3.3 | Induction of death is associated with alpha-hemolysin expression by NMEC

One of the major differences between NMEC RS218 and NMEC O18 is the possession of HlyA by NMEC.
RS218, but not NMEC O18. HlyA is associated with the induction of macrophage death during UPEC infection.\textsuperscript{21} To determine if NMEC strain-dependent cell death was limited to HlyA\textsuperscript{+} strains, we infected 6 additional HlyA\textsuperscript{−} strains and 7 additional HlyA\textsuperscript{+} strains. These strains were chosen to represent various phylogenetic groups and O-antigen groups (Table 1). As expected, all 6 β-hemolytic HlyA\textsuperscript{+} strains induced greater than 31\% cell death in macrophages, and this cell death was P2XR-dependent (Figure 3E). In contrast, all HlyA\textsuperscript{−} strains induced less than 3.5\% cell death in macrophages (Figure 3E), suggesting that HlyA is associated with, and likely responsible for, P2XR-dependent cell death. Interestingly, the one HlyA\textsuperscript{+}, but non-hemolytic strain, NMEC 42, induced ~10\% cell death, which is markedly lower than the >31\% cell death elicited by the HlyA\textsuperscript{+} strains that are β-hemolytic.

### 3.4 | In vivo inhibition of P2XRs significantly decreases survival of neonatal mice infected with NMEC RS218, but has no significant effect on neonatal mice infected with NMEC O18

To determine the role of purinergic receptors in vivo, we treated 3-day-old mice with BBG or PBS prior to infection with 5 × 10\(^3\) CFU NMEC RS218. We found that BBG treatment significantly decreased survival from 50\% to 7\%, and increased bacterial loads >1000 fold in the blood of NMEC
RS218-infected mice (Figure 4A,B). Similarly, we found that apyrase treatment markedly decreased the survival of neonatal mice infected with NMEC RS218 (Figure S5A,B), confirming that sensing of ATP by nucleotide receptors confers protection against NMEC RS218.

We next pre-treated neonates with BBG or PBS and infected these mice with NMEC O18, and found no difference in survival or bacteremia between the two groups (Figure 4C,D). To ensure that any potential protective effects of P2XRs were not masked by the increased virulence of NMEC O18 compared to NMEC RS218 (Figure 1), we next infected mice with 50 CFU NMEC O18. While infection with the lower dose of NMEC O18 did extend the median time to death of control animals (20 h for 5000 CFU vs. 24 h for 50 CFU), there was still no significant difference in survival between control pups and those treated with BBG (Figure 4E,F). These results suggest that P2XR activation plays a protective role in NMEC RS218 infection, but not NMEC O18 infection.

P2X7 is one of the most widely studied P2XRs and has been found to play a role in other E. coli infection models. Furthermore, BBG is often used at low doses as a P2X7-specific inhibitor. To determine if the effects of BBG in our model were due to specific inhibition of P2X7, we infected P2X7−/− macrophages with NMEC RS218. Interestingly, we found that there was a significant but modest difference in cell death between wildtype and P2X7−/− cells (Figure 5A). We also treated P2X7−/− macrophages with BBG and found a marked decrease in cell death among the BBG-treated cells (Figure 5B), indicating that the effect of BBG-treatment in our system is not solely due to inhibition of P2X7. To determine if this was also the case in vivo, we pre-treated P2X7−/− neonates with BBG prior to infection with NMEC RS218. Mice were euthanized 18 h post-infection, and tissues were collected to determine bacterial loads. We found that the bacterial burden was significantly higher (>100 fold) in the brains, blood, and peritoneum of P2X7−/− pups treated with BBG, but that there was no significant difference in bacterial loads in the spleen (Figure 5C–F). This suggests that the effect of BBG on outcomes of NMEC RS218 infection in neonatal mice is not solely dependent on specific inhibition of P2X7, and that other P2XRs either alone or in concert are likely responsible for the protective effects of P2XRs in our model. BBG can also antagonize P2X4. Indeed, in vitro we found that inhibition of P2X4 with Bay-1797 in P2X7 deficient cells had a partial effect on NMEC-RS218 induced cell death (Figure S4C), indicating that P2X4, P2X7, and potentially other P2XRs may respond to ATP and function in concert to protect neonates against NMEC RS218. In addition to P2XRs, BBG has been reported to inhibit voltage-gated sodium channels. However, we found that the treatment of macrophages with the voltage-gated sodium channel inhibitor QX-314 had no effect on RS218-induced cell death (Figure S4D).

While P2XRs can activate NLRP3, in vitro we found that NLRP3 was not required for P2XR-dependent cell death (Figure 2) induced by NMEC RS218. We further determined that NLRP3 is not essential for P2XR-dependent protection in vivo, as NLRP3−/− pups treated with BBG had significantly increased bacterial loads (~40–1000 fold) in the brain, peritoneum, and spleen compared to PBS-treated pups (Figure S6A–D) following NMEC RS218 infection. It is important to note that bacterial loads in the blood were not significantly different between groups (Figure S6B), which could indicate a partial dependence on NLRP3 in vivo.

### 3.5 The protective effect of P2XR signaling requires the presence of macrophages, but not neutrophils

We found that bacterial loads in the brain, blood, peritoneum, and spleen were similar between PBS- and BBG-treated pups 6 h following NMEC RS218 infection (Figure S7A–D), and only ~10-fold higher in BBG treated animals at 12 h post-infection (Figure S7E–H). We, therefore, used these time points to assess cytokine and chemokine production in the peritoneum to evaluate early immune responses that may contribute to the markedly different bacterial levels found 18 h after infection. At 6 h post-infection, when bacterial counts were similar, control mice had significantly higher IFN-γ, IL-1β, IL-6, IL-17, CXCL9, and CCL2 (Figure 6A–F), indicating a diminished early pro-inflammatory response in BBG treated animals. By 12 h post-infection, BBG-treated mice had similar levels of IFN-γ, IL-1β, IL-6, IL-17, and CXCL9, and significantly elevated CCL2, CXCL1, CCL3, and TNF-α (Figure 6). However, this was not completely unexpected since by this point bacterial loads were significantly higher in BBG-treated animals and this increased bacterial burden likely contributes to increased cytokine levels. No differences were noted in IL-10 or IL-4 production at either time point (data not shown).

Neutrophils are one of the first cells recruited to the site of infection and have been found to be protective during NMEC infection in both adult and neonatal mouse models. Furthermore, P2XRs play a key role in neutrophil activation and function, specifically playing a role in chemotaxis, extravasation, cytokine release, and prolonging cell survival. IL-1β, IL-6, and IL-17 were decreased in the lavage samples from BBG-treated mice at 6 h post-infection (Figure 6B–D), and are all associated with recruitment of neutrophils to the site of infection. To determine if P2XR-dependent protection requires
FIGURE 4  P2XR Signaling is protective against infection with NMEC RS218, but not NMEC O18, in neonatal mice. Three-day-old mice were treated with either PBS or BBG (50 mg/kg) 2 h prior to infection with either 5000 CFU NMEC RS218 (A and B) (n = 12–13/group), 5000 CFU NMEC O18 (C and D) (high-dose, n = 9/group), or 50 CFU NMEC O18 (E and F) (low-dose, n = 10/group). Blood was taken 18 h post-infection (B, D, and F) and mice were monitored for survival (A, C, and E). Three PBS-treated and 2 BBG-treated mice in the high-dose NMEC O18 infection group were dead by 18 h post-infection, and blood could not be collected. Data are combined from 2 or 3 independent infections. The solid lines represent the sample median. The dashed lines represent the limit of detection of CFU detection. Significance is set at p < .05.
neutrophils, we depleted neutrophils from neonatal mice prior to PBS or BBG treatment and subsequent infection with NMEC RS218. Interestingly, following neutrophil depletion, BBG-treated mice still had significantly higher bacterial loads (∼50–1000 fold) in blood, peritoneal lavage, and spleen at 18 h post-infection (Figure S8), suggesting that neutrophils are not essential for the protective effect of P2XR activation during NMEC RS218.

Macrophages and monocytes are also known to be recruited and activated in response to P2XR signaling, CCL2, which we found to be diminished in BBG-treated mice at 6 h post-infection (Figure 6F), is an important chemoattractant for monocyte migration to the site of infection.67 We have also shown that P2XRs induce macrophage cell death during in vitro infection with NMEC RS218 (Figure 3), suggesting that P2XRs on macrophages are activated by NMEC RS218 infection. In addition, we found that depletion of macrophages with clodronate liposomes enhances the susceptibility of neonatal mice to colonization with NMEC RS218 by ∼200–4000 fold (Figure 7A–D). To determine if P2XR-dependent protection required macrophages, we treated pups with clodronate liposomes one day prior to PBS or BBG treatment and NMEC RS218 infection. In clodronate-treated pups, we did not see an effect of BBG on bacterial loads in the lavage or spleens (Figure 7G,H), indicating that macrophages are required for P2XR-dependent protection. Interestingly, in clodronate-treated animals, we also found that the blood and brains from BBG-treated pups had ∼10 fold significantly lower bacterial loads than PBS-treated mice (Figure 7E,F). This could indicate a tissue-dependent deleterious role of P2XR signaling in the absence of macrophages.

To determine whether the protective effect of P2XR signaling was due to stimulation of increased phagocytosis or bacterial killing by macrophages we next treated macrophages with PBS or BBG prior to infection with NMEC RS218. There was no difference in the ability of macrophages to uptake or kill the bacteria at any time point assessed (Figure S9A–C), suggesting that P2XRs are not inducing protection by improving the phagocytic or bacteria-killing capacity of macrophages.

**Figure 5** P2X7-R is not essential for the protective effect of P2XR activation in vivo following infection with NMEC RS218. Macrophages harvested from wildtype (WT) or P2X7-R−/− mice were infected with NMEC RS218. Supernatant was collected 5 h post-infection to assess cell death using an LDH assay (A). In (B), P2X7-R−/− cells were pre-treated with PBS or BBG (100 µM) ~1 h prior to infection. Data are representative of 2 independent experiments with 3 wells/groups. *p < .05. The error bars represent SD of the mean. (C–F) 3-day-old P2X7−/− mice were pre-treated with PBS or BBG (50 mg/kg) ~2 h prior to infection with 5000 CFU of NMEC RS218. Mice were euthanized 18 h post-infection and brain homogenate (C), blood (D), peritoneal lavage (E), and spleen homogenate (F) were plated on MacConkey agar to determine bacterial loads. n = 12–13 per group. Data are combined from 3 independent infections. The solid lines represent the sample median. The dashed lines represent the limit of detection CFU detection. Significance is set at p < .05
**FIGURE 6** Treatment with BBG alters inflammatory cytokine profiles in peritoneal lavage following NMEC RS218 infection. Three-day-old mice were pre-treated with PBS or BBG (50 mg/kg) ~2 h prior to infection with 5000 CFU of NMEC RS218. Mice were euthanized 6 h or 12 h post-infection and peritoneal lavage fluid was assessed for inflammatory cytokines using a Luminex magnetic bead assay. Data are combined from 3 independent infections. The solid lines represent the sample median. Significance is set at $p < .05$.

**FIGURE 7** The protective effect of P2XR signaling during NMEC RS218 infection requires macrophages and monocytes. Two-day-old wildtype mice were depleted of macrophages/monocytes with clodronate liposomes or empty liposomes and then infected with 5000 CFU of NMEC RS218 on post-natal day 3. Mice were euthanized 16–18 h post-infection and brain homogenate (A), blood (B), peritoneal lavage (C) and spleen homogenate (D) were plated on MacConkey agar to determine bacterial loads. Data are combined from 4 independent infections. $n = 12–13$ per group. In (E–H) mice were treated with clodronate liposomes on post-natal day 2 and then pre-treated with PBS or BBG (50 mg/kg) ~2 h prior to infection with NMEC RS218 on post-natal day 3. Brain homogenate (E), blood (F), peritoneal lavage (G), and spleen homogenate (H) were collected 16–18 h post-infection. $n = 11–12$ per group. Two PBS-treated mice and 1 BBG-treated mouse were found dead at the necropsy and blood could not be collected. The solid lines represent the sample median. The dashed line represent the limit of detection of CFU detection. Significance is set at $p < .05$. 
Despite significant variability in virulence gene possession in NMEC strains, relatively little has been done to assess the impact of this variability on the pathogenesis of NMEC infection. To date, much of the focus has been on factors associated with crossing the BBB. Many determinant factors have been identified, including the K1 capsule, OmpA, FimH, and CNF-1. However, in order to cause meningitis, NMEC must first evade the host immune response to reach a threshold bacteria. The most well-studied NMEC virulence factor in this context is OmpA. Expression of OmpA is associated with subverting the complement cascade and suppressing the bacterial killing ability of macrophages and neutrophils. However, only 66% of NMEC isolates possess OmpA, and this is not significantly different from the expression of OmpA by commensal human fecal *E. coli*, suggesting that the presence of OmpA is not the sole determinant factor for NMEC virulence. Many other virulence factors have the potential to modulate the immune response to NMEC infection including HlyA, which has been studied in the context of uropathogenic *E. coli*, but not in NMEC. To that end, we assessed the role of P2XR signaling, which we found to be associated with HlyA expression, on the outcomes of NMEC infection in neonatal mice.

We found that NMEC RS218, an HlyA-expressing NMEC strain, induced P2XR-dependent cell death in vitro while NMEC O18, an HlyA- NMEC strain, did not (Figures 1 and 3). P2XR activation has previously been associated with HlyA expression by UPEC. We, therefore, infected macrophages with 13 additional HlyA- or HlyA- NMEC strains to determine whether the same was true for NMEC infection. We found robust P2XR-dependent cell death was induced by all 7 additional HlyA- NMEC strains, while HlyA- strains induced little to no cell death (Figure 3). NMEC 42 is HlyA-, but does not cause appreciable hemolysis on blood agar (Table 1). This may represent a defect in regulators of HlyA function. For example, the cof gene has been identified as a regulator of HlyA activity in the UPEC strain CFT073. Deletion of the cof gene resulted in significantly decreased HlyA secretion, as well as significantly reduced cell lysis and IL-1β secretion. Similarly, we found that NMEC 42 induced only a third as much P2XR-dependent cell death as did the hemolytic HlyA+ NMEC strains (Figure 3E), further suggesting an association between HlyA activity and P2XR activation.

Activation of P2XRs results in K+ efflux from the cell and is known to activate the NLRP3 inflammasome, leading to the release of IL-1β and cell death via pyroptosis. However, we found that NMEC RS218 induced NLRP3-dependent IL-1β secretion does not require P2XR activation, and that P2XR-dependent cell death does not require NLRP3 activation (Figures 2 and 3). In host cells, HlyA forms a large pore 2.0–2.6 nM in diameter that forms a channel for ATP release and also causes membrane damage leading to K+ efflux and NLRP3-dependent IL-1β production.

Therefore, our findings that BBG inhibits ATP-dependent cell death in response to NMEC RS218 without affecting NLRP3/K+ dependent IL-1β production (Figures 2 and 3) suggest that BBG inhibits nucleotide receptor signaling without directly affecting the generation of the HlyA pore. BBG also inhibited gasdermin-D-dependent cell death in response to LPS/nigericin (Figures 2 and 3), perhaps because BBG inhibited P2X-dependent K+ efflux. Both NMEC RS218 and LPS/nigericin-induced cell death were inhibited by glycine (Figure 2). Interestingly, glycine was recently reported to prevent the clustering of ninjurin, a newly identified executor of plasma membrane rupture that mediates cell lysis in pyroptotic, apoptotic, and necrotic forms of cell death. Therefore, in the future, we will investigate the role of ninjurin in P2X-dependent cell death.

While P2XR-dependent cell death and NLRP3-dependent IL-1β production appear to be uncoupled during NMEC RS218 infection, it has been reported that lower expression of HlyA by UPEC strains is associated with cell death partially dependent on NLRP3, while high expression of HlyA resulted in NLRP3-independent cell death. Therefore, it is possible that NMEC RS218 expresses high levels of HlyA that potentially lead to robust ATP release and that this is the reason for the uncoupled NLRP3 and P2XR responses. However, further studies would be needed to correlate the requirement of NLRP3 for cell death with HlyA expression levels in NMEC strains.

To test the role of bacterial strain-dependent P2XR activation during NMEC infection in vivo, neonatal mice were treated with the P2XR inhibitor, BBG, prior to infection with NMEC RS218. BBG-treated mice had significantly increased bacteremia at 18 h post-infection and significantly decreased survival (Figure 4A,B). Similar effects were observed when mice were treated with apyrase, which hydrolyzes ATP (Figure S5). In contrast, inhibition of P2XRs had no impact on the outcome of NMEC O18 infection (Figure 4C–F). This finding, in combination with the observation that NMEC O18 infection results in significantly higher bacterial burdens than NMEC RS218 infection (Figure 4A), suggests that NMEC strain-dependent activation of P2XRs is protective during NMEC infection, and factors that activate this pathway, such as HlyA, may potentially be detrimental to the virulence of NMEC.

The effect of P2XR signaling has often been attributed to its role in the activation of NLRP3 and subsequent
production of IL-1β.\(^5\) However, neonates have a diminished NLRP3 response compared to adults.\(^7\) We also previously showed that IL-1 signaling is only protective in neonatal mice in the absence of iNOS\(^5\) and that P2XR activation and NLRP3 activation appear to be uncoupled during in vitro NMEC RS218 infection (Figures 2 and 3). It is, therefore, unlikely that NLRP3 activation is responsible for the P2XR-dependent protection in response to NMEC RS218 infection. To confirm this in vivo, we treated neonatal NLRP3\(^{−/−}\) mice with BBG prior to infection with NMEC RS218. As expected, P2XR-dependent protection was not completely dependent on NLRP3 activation (Figure S6).

P2XR activation can have a number of effects on various innate immune cells in vivo, including both neutrophils and macrophages.\(^29\) We found that BBG-treated neonates had significantly decreased IL-1β, IL-6, and IL-17 in peritoneal lavage fluid following NMEC RS218 infection (Figure 7B–D), all of which are associated with neutrophil chemotaxis.\(^64–66\) However, in the absence of neutrophils, we still found that BBG-treated pups had significantly higher bacterial loads, indicating that the protective effect of P2XR does not require neutrophils (Figure S8).

Our peritoneal cytokine analysis also showed significantly decreased CCL2 in BBG-treated neonates early in infection (Figure 6F), and we observed that macrophage depletion enhanced the susceptibility of neonatal mice to infection (Figure 7A–D). These results, along with our finding that P2XRs are activated on macrophages in vitro (Figure 3), suggested that macrophages could potentially be involved in P2XR dependent protection against NMEC RS218 in neonates. In macrophage-depleted animals, BBG treatment did not significantly alter bacterial loads in the peritoneum or spleens of NMEC RS218-infected pups (Figure 7G,H) indicating that P2XR signaling is only protective in the presence of macrophages. Interestingly, BBG-treated mice had significantly decreased bacterial loads in the brain and blood (Figure 7E,F). This could represent a deleterious effect of P2XR signaling in the absence of macrophages or could be indicative of a site-specific difference in the role of P2XR signaling on infection. We also found that P2XR signaling had limited effects on bacterial uptake and no significant impact on bacterial killing by macrophages (Figure S9A–C), suggesting that modulation of these functions is likely not the mechanism of P2XR-dependent protection. P2XRs are also known to play a role in monocyte/macrophage chemotaxis and cytokine release.\(^29\) We will, therefore, address the effects of P2XRs on macrophage migration and cytokine production in future studies.

While we found that depletion of macrophages with clodronate enhanced the susceptibility of pups to NMEC RS218 (Figure 7A–D), a previous study found that depletion of macrophage with α-carrageenan decreased bacterial loads in neonates following i.n. infection with a rifampicin-resistant derivative of NMEC RS218.\(^7\) This effect was attributed to OmpA-dependent evasion of macrophage bactericidal properties, allowing the macrophages to become a niche for bacterial replication.\(^70,78\) This study used mice of the same age and genetic background as were used in our study. Therefore, our differing results may indicate a difference in macrophage depletion methods (α-carrageenan vs clodronate liposomes) or differences in the macrophage response depending on the route of infection (intranasal vs. intraperitoneal). Treatment with α-carrageenan has also been shown to enhance macrophage-dependent TNF-α and IL-6 secretion and increase mortality in response to LPS-induced septic shock.\(^79\) Alternatively, treatment with clodronate liposomes induces macrophage apoptosis and is not believed to activate macrophages prior to depletion.\(^75\) Therefore, the differential effects of clodronate and α-carrageenan on inflammation may explain the discrepancy between our study and previous work.

Taken together, the data presented here indicate that P2XR activation confers strain-dependent protection against NMEC infection and that macrophages, but not neutrophils are required for this protection. Our data further suggest that this strain-dependent effect is due to the expression of HlyA. More research is required to determine how P2XRs mediate macrophage-dependent protection (i.e., via increased recruitment or activation), as well as what specific P2XRs, are involved, and if treatment with P2XR agonists would improve outcomes of infection with HlyA\(^−\) NMEC strains. Nevertheless, these data clearly show that variability in virulence factor profiles between NMEC strains can have a significant impact on the immune response. Based on these findings, it is important that future research takes this into account by utilizing multiple NMEC strains when investigating the pathogenesis of NMEC infection. These findings may also lead to advances in diagnostic and prognostic procedures, as identification of specific virulence factors, such as HlyA, from culture specimens may give insight into the expected course of infection. Alternatively, future research may determine biomarkers associated with P2XR activation which could also serve as prognostic indicators, and potentially aid in the development of improved treatment plans for human neonates infected with NMEC.

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None noted.

AUTHOR CONTRIBUTIONS
Catherine A. Chambers and Jerod A. Skyberg designed research. Catherine A. Chambers, Alexis S. Dadelahi, and Charles R. Moley performed research. Rachel M. Olson and Catherine M. Logue contributed new reagents or analytic tools. Catherine A. Chambers and Jerod A. Skyberg analyzed data. Catherine A. Chambers and Jerod A. Skyberg wrote the paper.

DATA AVAILABILITY STATEMENT
Not applicable.

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SUPPORTING INFORMATION

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