INVESTIGATING AGE- AND VIRULENCE FACTOR- DEPENDENT INNATE IMMUNE ACTIVATION DURING NEONATAL MENINGITIS ASSOCIATED E. COLI INFECTION

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DEDICATION

I would like to dedicate this work to my wonderful parents, John and Dorothy Chambers, who have always believed in me even when I struggled to believe in myself.
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Footnotes:

A. Chapter III. This work was published in *Immunology and Cell Biology* in 2021 with Catherine A. Chambers as the first author, followed in authorship order by Carolyn A. Lacey, Dana C. Brown, and Jerod A. Skyberg. CAC conceived the project, designed, performed and analyzed experiments, and wrote the manuscript. CAL assisted with experiments and methodology. DCB assisted with experiments and methodology. JAS conceived of the project, designed, performed and assisted with experiments, and edited the manuscript.
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ABSTRACT

Neonatal meningitis-associated *Escherichia coli* (NMEC) is a leading cause of early-onset sepsis and meningitis. While current antibiotic protocols have significantly reduced mortality associated with neonatal sepsis and meningitis, surviving infants are at a significantly increased risk of developing life-long neurologic impairment compared to healthy infants. This continued risk of lasting neurologic damage along with a recent rise in antibiotic resistant NMEC strains has precipitated a need for new therapeutic strategies. Targeted immune-based therapeutics may serve as desirable adjunct therapies; however, there are many challenges to their development. The neonatal immune system is immature compared to the immune system of adults, leading to an increased risk of infection. Here we assess the role of IL-1 secretion, which is known to be diminished in neonates, in the pathogenesis of a murine model of NMEC infection. We further highlight one potential mechanism for decreased IL-1 secretion during the neonatal period. To further complicate the development of immune-based therapeutics aimed at treating NMEC infection, NMEC strains can possess many different virulence factors with the potential to alter immune cell activation. While some of these factors, such as OmpA, have been well-characterized in the context of NMEC infection, many potential virulence factors have not. Here we show that the pore-forming toxin α-hemolysin activates purinergic receptors, leading to improved bacterial clearance and decreased mortality in a neonatal mouse model of NMEC.
infection. Together, the data presented here provides new insight into both neonatal immunity and the role of virulence factor-specific immune activation on the pathogenesis of NMEC infection, and may serve as a stepping stone for the development of not only new immune-based therapeutics, but also new diagnostic and prognostic tools for use during neonatal infection.
CHAPTER I

INTRODUCTION

Escherichia coli and its commensal niche

*Escherichia coli* are common bacteria of the gastrointestinal (GI) system of warm-blooded animals and reptiles, and are generally the most prevalent aerobic bacterium in the GI tract (1). *E. coli* are Gram-negative, rod-shaped bacteria belonging to the family *Enterobacteriaceae* (1, 2). They are facultative anaerobes, facilitating their survival in the gastrointestinal system, and may be flagellated and motile, or non-motile (2). The majority of *E. coli* strains ferment lactose, which can be used to identify them in mixed culture on MacConkey agar (2, 3).

The relationship between *E. coli* and its host organisms is generally considered to be commensal, with *E. coli* acquiring important nutrients and a favorable growing environment from the GI tract with little impact on the host. However, *E. coli* can also benefit the host environment by preventing overgrowth of other, potentially pathogenic, bacteria via the production of bacteriocins, among other mechanisms (1, 4, 5). *E. coli* are some of the earliest bacteria to colonize the human intestine, appearing in feces as early as one day of age (6). Infants generally become colonized with *E. coli* derived from the maternal GI tract (7); however, *E. coli* may also be acquired from other environmental sources including, hospital nursing staff (8).
*E. coli* has a core genome which is inherited clonally (1, 9, 10). Multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) of these conserved regions has identified four major phylotypes among *E. coli* strains: A, B1, B2, and D (1, 2, 11). The relative prevalence and abundance of these phylotypes within the GI tract varies between host species, with phylotype A being most abundant in the human gastrointestinal tract (1). The average *E. coli* genome contains over 4500 genes, of which only 2000 are part of the highly conserved core genome. The remaining genes are part of a highly variable, flexible genome, which is passed along via horizontal gene transfer into specific integration sites (1, 2, 10). Currently, more than 8000 genes have been identified within this flexible genome pool, including a number of virulence genes, leading to significant diversity among *E. coli* strains (1, 9, 10, 12). Serotyping can also be used to characterize *E. coli* strains based on clonally inherited surface antigens. Serotyping systems generally utilize the O (lipopolysaccharide), H (flagellar), and sometimes K (capsular) antigens to categorize *E. coli* (13, 14).

**Extraintestinal pathogenic *Escherichia coli***

Although *E. coli* are an important part of the normal GI microbiome, they can also be pathogenic. Pathogenic *E. coli* are often defined based on their location and the clinical syndrome they cause. Intestinal pathogenic/diarrhea-causing *E. coli* (IPEC) are a major contributor to diarrheal disease worldwide, and can be broken down further into six well-defined pathotypes: enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and diffusely adherent *E. coli* (2, 13). In contrast,
extraintestinal pathogenic *E. coli* (ExPEC) are *E. coli* that primarily cause disease outside of the gastrointestinal tract, and are among the leading causes of extraintestinal infection (11, 15, 16). ExPEC strains are also characterized by where they primarily cause infection and include the following: uropathogenic *E. coli* (UPEC), which are a common cause of urinary tract infection in humans; sepsis-associated *E. coli* (SEPEC); and neonatal meningitis-associated *E. coli* (NMEC), which is associated with sepsis and meningitis of newborn infants. ExPEC are facultative pathogens, meaning that while, unlike IPEC, they may act as commensal organisms within the GI tract, when they are introduced into other body systems such as the urinary tract or blood stream, they can cause severe disease (1, 5, 11). These bacteria generally have adaptations which allow them to subvert the immune response in order to colonize these other tissues (9, 17).

**Characterization/identification of ExPEC**

There is no single way to differentiate ExPEC from commensal populations (11, 18). Indeed, while most ExPEC fall within the B2 and D phylotypes, there is significant genetic overlap with commensal *E. coli* (11, 18, 19). Similarly, certain serotypes and sequence types (ST) are associated with ExPEC strains, but these factors alone are not enough to distinguish ExPEC from commensal *E. coli* (11, 18). Instead, identification of potential ExPEC generally relies on a combination of these factors along with identification of specific virulence factors. On average, ExPEC isolates express more virulence genes than commensal *E. coli* (17, 20), and virulence factors associated with ExPEC infection are distinct from factors associated with IPEC virulence (5). Interestingly, the acquisition of these virulence
factors is thought to be coincidental, as there is no direct selective pressure on *E. coli* to become extraintestinal pathogens. Instead, many of these virulence traits are involved in pathways that make them more fit within their intestinal niche (e.g., transcription regulation, iron metabolism, adhesion, LPS biosynthesis, and peptide-polyketide hybrid synthesis systems) (18), which contributes to the difficulty in differentiating potential ExPEC isolates from commensal *E. coli*.

**Neonatal bacterial meningitis**

Bacterial meningitis is a severe, life-threatening manifestation of infection (21, 22). Specifically, meningitis refers to inflammation of the meninges, the membrane covering the brain and spinal cord, in direct response to bacterial colonization or the presence of bacterial products (21). Neonates, defined as infants under 28-90 days of age, have the highest incidence of meningitis of any age group (23). Bacterial meningitis, as determined by culturing of bacteria from the cerebrospinal fluid (CSF), affects approximately 0.3 in every 1000 live births in developed countries, and prevalence has been estimated at 0.8-6.1 cases per 1000 live births in developing countries (22). These are likely underestimations due to a variety of factors including the lack of CSF culture in many cases of bacteremia/sepsis and the administration of antibiotics prior to CSF collection (22).

Neonatal bacterial meningitis can be categorized into early- or late-onset based on the temporal presentation of clinical signs and isolation of bacteria from the CSF. Early-onset infection is defined as onset prior to 3-7 days of life, and late-onset is defined as onset after a week of age (22, 23). Age of onset can be suggestive of the mode of acquisition, with early-onset cases more likely acquired
vertically from the mother and late-onset infections more likely to be community-acquired (22, 23). Relatively few pathogens are responsible for the vast majority of neonatal meningitis, and these include *Streptococcus agalactiae* (Group B Strep; GBS), *E. coli*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, coagulase-negative *Staphylococcus* spp., *Staphylococcus aureus*, and *Klebsiella* spp. (21, 22). These organisms can also generally be categorized into those predominantly associated with early-onset infection (GBS, *E. coli*, *L. monocytogenes*, and *S. pneumoniae*) and those most frequently associated with late-onset infection (coagulase-negative *Staphylococcus*, *S. aureus*, and *Klebsiella*).

Premature birth and very low birth weight (VLBW; <1500 g at birth) are among the biggest risk factors for developing neonatal bacterial meningitis (22, 23). This is thought to be due to insufficiencies in the premature infant’s innate and adaptive immune responses (22-24). Other risk factors include maternal GBS colonization, premature or prolonged rupture of fetal membranes, invasive fetal monitoring, prolonged hospitalization, and the need for post-natal devices like shunts or catheters (22, 23). Many of the factors overlap and likely contribute to the increased risk of infection among premature infants who are more likely to be hospitalized for an extended period following birth.

In most cases, neonatal bacterial meningitis is secondary to bacteremia (22, 25, 26). This is consistent with data from animal models, where the percentage of animals that develop meningitis is directly proportional to the bacterial load in the blood (27, 28). Bacteria may enter the blood via several routes, often associated
with the risk factors listed above, including direct introduction by catheters, shunts or other invasive devices, or crossing of weakened skin or mucosal barriers (22, 23). During the birthing process, infants may inhale bacteria from the maternal rectovaginal region. These bacteria can then colonize the lungs, resulting in pneumonia, and/or cross the mucosal barriers to colonize the blood stream leading to bacteremia, sepsis, and potentially meningitis (23). Once in the blood, there are several mechanisms bacteria can use to cross the blood-brain barrier (BBB), which normally protects the brain from invading organisms (26). Some bacteria, such as *E. coli*, GBS, and *S. pneumoniae*, cross the BBB transcellularly. These bacteria are able to interact with specific receptors on the surface of BBB endothelial cells in order to enter and move through the cells and into the CSF without disruption of intercellular tight junctions (26). Organisms like *L. monocytogenes* can also enter the CSF via a Trojan-horse mechanism, which involves hiding in infected phagocytes to move across the BBB (26).

The primary way to diagnose neonatal bacterial meningitis is through Gram staining and culture of the CSF, which is collected via lumbar puncture (LP) (22, 23). However, the decision of if and when to perform an LP may not be straightforward. The clinical presentation of neonatal meningitis is frequently non-specific, with infants presenting with fever, poor feeding, vomiting, diarrhea, and/or lethargy (21-23). Often, clear clinical signs of meningitis, such as convulsions, bulging fontanel, and nuchal rigidity, do not manifest until late in infection, and are poor prognostic indicators (22). Additionally, while the bacteria reach the brain via a hematogenous route and approximately 30% of neonatal sepsis patients also
have concurrent meningitis (22, 29), 15-55% of neonates with confirmed bacterial meningitis have negative blood cultures at the time of diagnosis (22, 23, 30-32). To further complicate diagnosis, the initiation of antibiotics prior to LP, while frequently recommended, can result in false negative cultures (23). CSF indices such as leukocyte counts and pleocytosis may provide more insight into the possibility of central nervous system infection. However, these indices are poorly defined in neonates, and normal values may vary based on the age of the infant (22). Different techniques such as PCR testing of blood or CSF are becoming more common tools in the diagnosis of neonatal bacterial meningitis (23). However, there is a continued interest in development of new, more precise, diagnostic and prognostic tools.

**Neonatal meningitis-associated *E. coli***

Neonatal meningitis-associated *E. coli* is the ExPEC pathotype most frequently associated with sepsis and meningitis in neonates. NMEC is generally associated with early-onset sepsis and meningitis, and is considered the second-leading cause of neonatal bacterial meningitis (behind GBS), and the leading cause in premature and VLBW infants (22, 33). It is estimated that NMEC accounts for approximately 30% of early-onset cases of neonatal bacterial meningitis (22, 33). As peripartum antibiotic treatment for GBS becomes more prevalent, some hospitals have reported a relative rise in NMEC infection (34-36).

While there is no single determining factor that defines all NMEC strains, there are some virulence traits overrepresented within confirmed NMEC isolates. The K1 antigen, which is often used as a marker of NMEC strains, is present on
70-80% of tested isolates (20, 37). Certain O-groups are also overrepresented in NMEC isolates, with O18 being the most common (20, 38). Additionally, H7 is the most common H type associated with NMEC isolates, although it is not unique to NMEC (20). However, none of these serologic markers are present in all NMEC isolates, nor do they completely separate NMEC isolates from non-pathogenic \textit{E. coli} (11). On average, NMEC isolates express more virulence-associated genes compared to fecal commensal \textit{E. coli} (20). Furthermore, some of these factors, which will be described in more detail later, have been associated with the ability of NMEC strains to colonize the blood and invade the central nervous system (CNS). However, there is significant variability in virulence factor profiles between NMEC strains (20), which makes identification of potential NMEC strains difficult.

**Therapeutics**

Currently, treatment of NMEC infection and other forms of neonatal bacterial meningitis relies on the use of antibiotics and supportive care. It has been shown that increased time to bacterial clearance negatively impacts outcomes (22, 39). Therefore, empirical antibiotic therapy is generally started as soon as bacterial sepsis or meningitis are suspected (21-23). While these empirical antibiotic protocols may be influenced by many different patient and environmental factors, such as common bacterial resistance profiles within a specific hospital setting, common antibiotics used to treat suspected neonatal meningitis include amoxicillin and a third-generation cephalosporin, such as cefotaxime (22, 23). An aminoglycoside, such as gentamicin, may be added when cephalosporin-resistant Gram-negative organisms are suspected (22, 23).
While improvements to antibiotic protocols have significantly decreased mortality rates associated with neonatal bacterial meningitis, they are still far from perfect. In addition to a continued mortality rate of 10% in antibiotic-treated patients, antibiotic regimens have not significantly affected the rates of neurologic deficits in surviving infants (22, 40, 41). One study found that at 9 years of age, 9-15% of children that had meningitis during the neonatal period had severe neurodevelopmental outcomes which included cerebral palsy, significant learning disabilities, and the need for special education services. A further 10% of these children had moderate outcomes defined as mild cerebral palsy, mild learning problems, or sensorineural hearing loss (41). Survivors of neonatal meningitis may also display impaired motor function and are at an increased risk of developing seizure disorders (22, 41). Studies also suggest that prematurity and VLBW could compound the risks of severe impairment (41, 42). Although certain adjunct therapies, like corticosteroids, are recommended in other age groups to decrease the risk of neurologic damage, very few studies have addressed the use of these therapeutics during the neonatal period and they are therefore currently not recommended for these infants (23).

**Antibiotic resistance**

In recent years, the rise in antibiotic resistance has become a concern across the medical community. Among NMEC strains, the rate of ampicillin resistance has been reported as high as 78% (43, 44). One study at Oklahoma University Children’s Hospital found that among 43 *E. coli* isolates from bacteremic neonates, 67% were resistant to ampicillin and 14% were resistant to gentamicin.
Others have found that approximately 11% of neonatal infections are resistant to third-generation cephalosporins, and many of these cases were also resistant to ampicillin (45, 46). More alarming, is the emergence of multidrug-resistant strains which have been isolated from neonates with bacteremia and/or meningitis (44, 47-49). In the best-case scenarios, these antibiotic-resistant bacteria may lead to a delay in administration of antibiotics to which the bacteria are susceptible and subsequent extended time to bacterial clearance. In the worst-case scenario, these delays or an inability to provide appropriate antibiotics may lead to higher mortality rates among neonates infected with one of these multidrug-resistant strains. This, along with the high incidence of continued neurologic impairment following antibiotic therapy, has precipitated an increased need for new therapeutic strategies, including strategies to promote more efficient bacterial clearance by the host-immune response while limiting immune pathways associated with excessive tissue damage. Development of these types of therapeutics requires an in-depth knowledge of host-pathogen interactions.

The neonatal immune system

One important consideration when performing research aimed at understanding neonatal disease is the inherent differences in the neonatal immune response compared to that of adults. The neonatal immune system has historically been described as being “immature” or “deficient” compared to the immune system of adults. More recently, it has become clear that the neonatal immune response is more complicated than originally believed. Under certain conditions, neonatal mice and humans can develop a competent immune response similar to that of
adults; however, under other conditions the neonatal immune response appears
deficient or absent (50). While not technically deficient, the neonatal immune
response in mice is strongly skewed towards a tolerogenic T H2 response (51-53).
Alternatively, the T cell responses in human neonates are not clearly skewed
towards T H2; however, they are significantly diminished in many cases when
compared to adults (50, 54). B cell responses are also altered in the neonatal
period. Antibody production is generally delayed and short lived with lower peak
production and lower antigen-affinity (50, 55). However, similar to T cell responses,
evidence suggests that under certain conditions, neonatal B cells can produce a
robust antibody response similar to that of adult cells (50). Overall, the general
propensity of neonates to have a diminished adaptive response poses a challenge
in development of effective vaccines for neonates, and also complicates our
understanding of the neonatal response to infection. While these differences in the
neonatal adaptive immunity may be explained in part by decreased numbers of
adaptive immune cells and/or alteration of adaptive-cell-intrinsic factors during the
neonatal period, there is also significant evidence that adaptive-cell-extrinsic
factors may also be responsible for these differences, with alterations in innate
immune antigen presentation and cytokine secretion frequently cited as potential
sources of variation (50, 53, 56).

The innate immune system provides a nonspecific first line of defense
against potential pathogens via physical barriers, innate humoral immune
components, and cell-mediated pathogen-killing, all of which are altered in
neonates (56). Physical barriers, such as the skin and mucous membranes, exist
to limit introduction of potential pathogens into the body. In adults, the acidic pH of skin, and sebaceous secretions on the skin surface help to prevent over-colonization by pathogenic bacteria (56). In contrast, neonatal skin has a neutral pH for the first several days of life and the sebaceous lipid layer is limited, resulting in increased permeability (56). This is exacerbated in premature infants, who also lack the protective vernix caseosa, a lipid-rich material that develops to coat fetal skin in the third trimester of pregnancy to aid in water retention and prevention of bacterial invasion (57, 58). The mucosa of the GI and respiratory tracts also play extremely important roles in preventing pathogen entry into the body (56). Similar to skin, several protective aspects of these mucosal surfaces are diminished in neonates, including decreased acidity of the GI tract, which is thought to aid in the colonization of commensal organisms (56). Counterintuitively, higher expression of pathogen recognition receptors on the GI epithelium of premature infants leads to increased potential for bacterial invasion due to excessive inflammation and loss of barrier integrity (58, 59).

The innate humoral immune response includes antimicrobial peptides, naturally occurring antibodies, and the complement cascade (60, 61). The complement system is made up of a series of proteins in the serum which can be activated in order to either opsonize bacteria for improved uptake by phagocytic cells, or directly lyse the bacteria via formation of the membrane attack complex (60, 61). Interaction of complement components is also important for induction of inflammatory response, including induction of chemotaxis and cytokine production by macrophages and neutrophils, as well as activation and function of B cells in
response to both T cell-dependent and -independent antigens (50, 62, 63). Bacteria which have developed mechanisms to evade the antimicrobial properties are considered “serum-resistant”. Traits that impart serum resistance on *E. coli* are thought to be important virulence factors for NMEC and SEPEC (61, 64-66). Neonates are known to be deficient in several elements of the complement cascade, including C3, C4bp, and C9 (56, 61, 67-69). Insufficient production of these components by neonates not only leads to decreased direct protection against bacteremia, but may also be involved in diminished innate and adaptive cell responses (62).

The cellular component of the innate immune system is also known to be altered in neonates. Neonates, and premature infants in particular, have lower numbers of dendritic cells (DCs) compared to adults. DCs from neonates also express lower levels of MHC-II, and the costimulatory molecules, CD80 and CD86, leading to decreased T cell activation, which may be responsible for a decreased response to vaccination (53, 56, 70). DCs also secrete pro-inflammatory cytokines such IL-12p70, IL-1β, and IL-6 (56, 71); however, this response is diminished in neonates, and DCs from neonates, and especially premature infants, produce higher levels of the anti-inflammatory cytokine IL-10 (56). Monocyte and macrophage function is also altered in neonates. While neonates do appear to have similar numbers of monocytes to adults, like neonatal DCs, the monocytes cultured from human cord blood express lower levels of MHC-II, CD80, and CD86 (56). Neonatal monocytes also display decreased chemotaxis and phagocytosis in response to infection, and TLR stimulation results in cytokine production promote
TH17 and TH2 rather than the TH1 profile associated with TLR stimulation of adult cells (56, 58). The neutrophil response is also thought to be deficient in neonatal infection. This deficiency could in part be due to a decreased pool of neutrophils to draw from during acute inflammation (56, 72, 73). Interestingly, one study suggested that non-specific protection associated with BCG vaccination may be due to induction of emergency granulopoiesis resulting in a larger neutrophil pool in response to other pathogens (72). Neonatal neutrophils do however also display impaired trafficking to the site of infection and are less able to phagocytose bacteria (56, 58). Additionally, neutrophils from premature infants have diminished respiratory burst capability, leading to decreased killing of phagocytosed bacteria (74). Overall, several impairments in the innate immune response of neonates could contribute to the increased risk of infection.

Host-pathogen interactions during NMEC infection

Factors which aid in the ability of NMEC strains to cross the BBB have been studied in depth. E. coli require a threshold level of bacteremia before they can cross into the CNS; however, a high degree of bacteremia alone is not sufficient to induce CNS infection (25, 27, 75). While there is significant variability in the virulence profiles of NMEC isolates, several virulence traits have been demonstrated to play an important role in crossing the BBB. As E. coli cross the BBB via transcellular mechanisms, they must interact with endothelial cell proteins to facilitate entry into and through the BBB cells (26). The NMEC proteins FimH, OmpA, and cytotoxic necrotizing factor-1 (CNF-1) have all been shown to interact with human brain microvascular endothelial cells and facilitate crossing of the BBB.
(26, 76). However, none of these proteins are universally present on NMEC isolates (20). While FimH and OmpA are both present on the majority of NMEC isolates, CNF-1 was expressed on only 27.45% of the NMEC isolates examined in one study (20). FimH, and OmpA were also seen on a large percentage of human commensal fecal E. coli (20), suggesting their presence is not an indicator of pathogenic potential. The K1 antigen, found on 70-80% of NMEC isolates (20), has also been identified as a critical determinant for crossing the BBB (27). The K1 antigen is a 2,8-linked linear homopolymer of sialic acid, closely mimicking structures found on the cell surface of a number of adult and neonatal tissues, including human fetal neuronal tissue (77). This molecular mimicry, along with the ability of sialic acid to inhibit the alternative complement pathway, contribute to the ability of K1 E. coli strains to evade the host immune response (77).

Evidence suggests that the K and O groups, may also be important for the development of bacteremia by aiding in serum resistance (61, 64-66). In particular, O6, O18, and K1 were all associated with serum resistance, while O12 and K5 were not (64). Serum resistance has also been associated with binding of the bacterial protein OmpA to complement component C4 binding protein (C4BP). Once bound, C4BP acts as an inhibitor of the complement pathway by degrading C3b and C4b, preventing formation of membrane attack complex which executes the lytic function of the complement system (65, 66). While many studies have focused on the ability of ExPEC to evade complement-mediated lysis, it is important to note that deficiencies in the neonatal complement system allow bacteria normally killed by adult serum to cause disease in neonates (61, 67, 68).
Other age-dependent mechanisms have also been assessed as potential mechanisms for neonatal susceptibility to NMEC infection. Healthy human neonates produce significantly higher baseline levels of nitric oxide (NO) when compared to adults, and this difference is even more pronounced in premature infants (78). Neonatal mouse microglia, the resident macrophage-like cell of the brain, express higher basal levels of inducible nitric oxide synthase (iNOS), one of three synthases responsible for NO secretion from cells (79). Furthermore, in a study assessing microglial cell responses to lipopolysaccharide (LPS)-stimulation by age, neonatal microglial cells produced lower levels of cytokine but higher levels of NO compared to microglial cells from adults (80). Interestingly, iNOS deficient (NOS\(^{-}\)) neonatal mice or neonatal mice treated with an iNOS inhibitor are significantly less susceptible to NMEC infection than wildtype neonates (81), suggesting that NO production is detrimental to the outcome of NMEC infection. Another study demonstrated that increased concentrations of iron within the neonatal versus adult mouse peritoneum facilitated increased NMEC growth and dissemination (82), again suggesting that age-specific physiology contributes to neonatal susceptibility to infection.

Studies have also assessed interactions between NMEC strains and various innate immune cells and how these interactions affect the outcome of infection. The Prasadaraao lab has published numerous articles on the role of bacterial OmpA in pathogenesis of NMEC infection. They have demonstrated that OmpA\(^{+}\), but not OmpA\(^{-}\), \textit{E. coli} suppressed maturation/activation of DCs as determined by downregulation of important costimulatory molecules required for
antigen presentation and activation of naïve T cells (83). They further described that DCs infected with OmpA+ *E. coli* produced higher levels of TGF-β and IL-10, indicating a tolerogenic response and a possible means by which NMEC limit immune activation and bacterial clearance (83). In another study, they described an important interaction between NMEC strains and macrophages. They found that macrophage-depleted neonatal mice had significantly lower bacterial loads in the blood and decreased meningitis incidence compared to control mice following intranasal (i.n.) infection with an OmpA+ NMEC strain (84). They found that OmpA binding to macrophage FcγRI facilitates entry of *E. coli* into macrophages and mediates resistance to normal bactericidal activity, allowing the bacteria to multiply and leading to increased bacteremia (84, 85). The Prasadaraao group has also assessed the role of CNF-1 in NMEC infection. They found that the presence of CNF-1 limited uptake of bacteria by macrophages (86). Mice infected with CNF-1+ *E. coli* also displayed a higher degree of brain pathology compared to mice infected with an isogenic mutant *E. coli* lacking CNF-1 (86), again suggesting a role for specific virulence factors in the pathogenesis of NMEC infection.

Several studies have been done to determine the role of neutrophils in NMEC infection. One study of adult mice infected intracranially (i.c.) with an O18:K1:H7 *E. coli* strain reported that concomitant depletion of neutrophils with anti-Ly6G antibody (clone 1A8) from mice resulted in significantly increased bacterial loads in the brain and spleen as well as increased mortality (87). Another study found that neutrophil depletion of neonatal mice infected intraperitoneally (i.p.) with *E. coli* K1 lead to significantly decreased survival compared to isotype
antibody treatment (88). This study also found that neonatal mice with a simplified microbiome, induced by treatment of dams with antibiotics prior to parturition, had significantly fewer neutrophils and were more susceptible to *E. coli* K1 infection than neonates born to control dams (88). Interestingly, a third study, which looked at the role of neutrophils in the pathogenesis of neonatal i.n. infection with the well-characterized NMEC RS218 strain, found that neutrophil-depleted neonates were resistant to infection (89). This group did show that interactions between OmpA on the bacteria and gp96 on the surface of neutrophils suppressed oxidative burst and also confirm that NMEC RS218 was able to survive and multiply within neutrophils (89). These results could also be in part due to the use of anti-GR-1 antibody (clone RB6-8C5), which could potentially deplete other GR-1+ cells, including some monocyte subsets and DCs (90); however, in the earlier mentioned study of adult mice, treatment with RB6-8C5 resulted in even higher bacterial loads than 1A8-treated animals, suggesting an additive protective effect of the role of neutrophils and other GR-1+ cells (87). Therefore, the seemingly conflicting results regarding the role of neutrophils in NMEC infection could be due to bacterial strain-dependent effects, host age, route of infection, or some other yet-to-be-determined variable.

**Inflammasomes**

Inflammasomes are a group of intracellular, multimeric protein complexes which sense and respond to damage-associated molecular patterns (DAMPs) and/or pathogen-associated molecular patterns (PAMPs). These inflammasome complexes are generally comprised of a sensor molecule which recognizes one or
more specific PAMPs or DAMPs, an adaptor protein and an effector caspase (91-93). Sensor complexes include member of the nucleotide-binding oligomerization domain (NOD)- and leucine-rich repeat (LRR)-containing receptors (NLRs), the absent in melanoma-2 (AIM2)-like receptors, and pyrin-containing receptors (93). Despite having different activating stimuli based on the sensor, inflammasomes follow a similar downstream cascade once activated. Recognition of PAMPS/DAMPs leads to recruitment and oligomerization of the adaptor protein apoptosis-associated speck-like protein (ACS), with subsequent recruitment of pro-caspase-1. Pro-caspase-1 is then cleaved to its active form, and active caspase-1 cleaves both pro-IL-1β and pro-IL-18 to their bioactive forms, which can be secreted from the cell to initiate an inflammatory response. Caspase-1 also cleaves gasdermin D (GSDMD). Once cleaved, the N-terminal region of GSDMD polymerizes in the cell membrane to create a pore with an approximate internal diameter of 18 nm (94). It is known that cytokines, particularly IL-1β and IL-18, can be secreted via these pores (94-96). In addition to serving as a cytokine secretion system, the accumulation of GSDMD pores in the cell membrane can also lead to destabilization of the membrane and ultimately result in lytic cell death, termed pyroptosis (94-96).

The NLR family pyrin domain-containing 3 (NLRP3) inflammasome is one of the most widely studied inflammasomes to date. While NLRP3 has been shown to respond to a wide range of endogenous DAMPs and PAMPs, including uric acid, nucleic acids, ATP, pore-forming toxins, and bacterial cell wall components, it is thought that it is likely sensing a common downstream cell distress signal (97, 98).
One current hypothesis is that these stimuli each lead, either directly or indirectly, to cell membrane damage which allows for potassium ions (K\(^+\)) to escape the cell due to osmotic forces (97). It is this efflux of K\(^+\) that is believed to activate the NLRP3 inflammasome, as addition of K\(^+\) into the extracellular media blocks activation of NLRP3. In the canonical NLRP3 activation pathway, this damage and K\(^+\) efflux is attributed to a direct effect of the inciting factor leading to direct activation of NLRP3. A noncanonical pathway has also been described in which caspase-11, which recognizes intracytosolic LPS (92), is first activated and cleaves GSDMD. The subsequent GSDMD pores allow for K\(^+\) efflux and lead to a secondary activation of the NLRP3 inflammasome.

**Interleukin-1**

The interleukin-1 (IL-1) family of cytokines includes several important members like IL-1\(\alpha\), IL-1\(\beta\), IL-1Ra, IL-33, IL-18, IL-36, IL-37, and IL-38 (99). IL-1\(\beta\) and IL-1\(\alpha\) are inflammatory cytokines which bind and activate the same receptor (IL-1R1) (100). Pro-IL-1\(\beta\) is not constitutively expressed (101). Instead, transcription of pro-IL-1\(\beta\) is induced in inflammatory cells, including macrophages and neutrophils following activation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Following this “step 1” response, a second stimulus is required to cleave pro-IL-1\(\beta\) to its active form. This “step 2” response is generally attributed to inflammasome activation and caspase-1 cleavage (101). IL-1\(\alpha\) on the other hand, is constitutively expressed in many cell types, including non-inflammatory cells, although its expression can also be upregulated during inflammation (102). Furthermore, while IL-1\(\alpha\) may be cleaved in an activation step,
pro-IL-1α is also biologically active if released from the cell following cell damage or necrosis (103).

Once released from cells, IL-1α and IL-1β both bind to and activate IL-1R1, from here on referred to simply as IL-1R. IL-1R can be found on hematopoietic cells such as monocytes/macrophages, dendritic cells, neutrophils, T cells, and B cells. It is also expressed on some non-hematopoietic cells such as epithelial cells (100). Activation of IL-1R induces a broad range of downstream inflammatory effects through the adaptor protein MyD88, making it an important contributor to both infectious and non-infectious inflammatory conditions (100). Specifically, IL-1R activation is known to elicit release of inflammatory cytokines, induce synthesis of cyclooxygenase-2 (COX-2), phospholipase-A2, and iNOS, as well as increase production of reactive oxygen species (ROS). Systemic effects of these events include fever, lowered pain thresholds, vasodilation, and hypotension (99). IL-1 signaling also plays an important role in inflammatory cell recruitment via upregulation of adhesion molecules and induction of chemokines (99). IL-1-mediated neutrophil recruitment was suggested to be protective in a mouse model of GBS infection (104). This study found IL-1R−/− adult mice had significantly increased bacterial loads and significantly decreased survival compared to wildtype mice following GBS infection. Furthermore, IL-1R−/− mice had significantly decreased KC and MIP-1α, two important chemokines for recruitment of neutrophils, as well as fewer neutrophils in the blood and tissues following infection (104). IL-1 signaling has also been shown to be protective in S. pneumoniae meningitis (105).
While IL-1 can be important for clearing potentially harmful pathogens, IL-1 signaling may also lead to overzealous inflammation and detrimental effects on the host. In a model of *Toxoplasma gondii* infection, IL-1R<sup>−/−</sup> mice initially showed signs of increased liver and adipose tissue pathology, indicating a potential protective role in early infection. However, chronically infected IL-1R<sup>−/−</sup> mice displayed improved survival compared to wildtype animals, indicating a deleterious role of IL-1 in chronic *Toxoplasma* infection via IL-1-induced cachexia (106). Of particular importance in the context of neonatal infection and meningitis, excessive IL-1 signaling in the brain has also been linked to neurologic damage and the development of long-term neurologic deficits following meningitis (107) and also sepsis without direct infection of the brain (108).

**The neonatal NLRP3 inflammasome and IL-1 response**

Neonatal humans have altered NLRP3 activity compared to adults. It has previously been shown that both cord-blood-derived monocytes and peripheral monocytes from newborn infants secrete significantly less mature IL-1β in response to LPS stimulation than peripheral blood macrophages from adults despite similar levels of pro-IL-1β (109, 110). This defect in IL-1β secretion was even more pronounced in blood from premature infants (110). Furthermore, both term and pre-term infants were found to have significantly lower NLRP3 expression on blood monocytes (109), which could account for the diminished IL-1β response. However, the mechanisms behind diminished NLRP3 activation in neonatal cells has not been fully elucidated. A similar result was also found when comparing macrophages isolated from 9-12-days-old mice to those of adult mice (111).
addition to decreased IL-1 secretion, the juvenile mouse macrophages were also noted to secrete significantly less IL-6 compared to cells from adult mice. Interestingly, this decrease in inflammatory cytokine secretion was linked to significantly increased IL-10 production in juvenile mouse macrophages, and cells from juvenile IL-10\(^{−/−}\) mice did secrete similar levels of both IL-1\(β\) and IL-6 to macrophages from adult mice following LPS challenge (111). It is known that IL-10 can dampen NLRP3 expression and activation in mouse macrophages following chronic LPS exposure (112). However, more research is need to fully understand the relationship between age and NLRP3 activation.

The role of NLRP3 and IL-1 in UPEC infection

The role of inflammasomes and IL-1 have been thoroughly assessed in UPEC, another example of ExPEC infection. During UPEC infection, the NLRP3 inflammasome is induced by α-hemolysin (HlyA)-expressing *E. coli* and leads to secretion of IL-1\(β\) by both macrophages and neutrophils (113, 114). HlyA was also shown to induce cell death in infected macrophages. Interestingly, this cell death was found to be either NLRP3-dependent or -independent depending on the degree of HlyA expression (114). Interestingly, there are conflicting reports on the role of NLRP3 activation and IL-1\(β\) in the progression and outcome of UPEC infection.

Some studies have found a protective effect of IL-1\(β\) secretion against infection (115-117). Specifically, following a model of acute pyelonephritis using UPEC strain CFT073, IL-1\(β^{−/−}\) mice exhibited more severe histopathologic changes in the kidney following infection (116). Another study using CFT073 *in vitro*
demonstrated that the bacterial protein TcpC, directly interacts with both NLRP3 and caspase-1 to prevent cleavage and release of active IL-1β (117). Furthermore, they demonstrated that IL-1β−/− mice had significantly more bacteria in the bladder both three and seven days after intravesicular instillation of bacteria (117).

On the other hand, one study found that the presence of NLRP3 increased *E. coli* invasion of the uroepithelium (113). In another study, inhibition of IL-1β led to significantly decreased pathology following intravesicular instillation of bacteria (118). Interestingly, this group did not see a significant difference in pathology between IL-1β−/− mice and wildtypes; however, NLRP3−/− or ASC−/− mice had significantly worse pathology and higher bacterial loads. These mice also, counterintuitively, displayed hyperactive IL-1β processing due to a noncanonical MMP7 pathway. Inhibition of IL-1β significantly improved pathology scores and decreased bacterial loads in ASC−/− animals, suggesting that overexpression of IL-1β can actually prevent effective bacterial clearance and worsen tissue damage (118). A third study found that inhibition of NLRP3 in mouse macrophages not only significantly reduced IL-1β secretion from cells, but also significantly increased the antimicrobial properties of the neutrophils as determined by phagocytic ability, ROS production, and inhibition of bacterial growth (119), further suggesting that NLRP3 activity can be detrimental during UPEC infection. These conflicting results suggest that the role of NLRP3 and IL-1β in UPEC infection is likely dependent on a complex interaction of factors including chronicity of infection, bacterial strain, infectious dose, host and bacterial genetics, and the degree of NLRP3
activation/IL-1β release. These results further suggest the need for continued research into the role of inflammasomes and IL-1 during ExPEC infection.

**P2X receptors**

Nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), can be released from cells under physiologic conditions via cell membrane pores and released from granules (120, 121). These extracellular nucleotides then bind to and activate a group of purinergic nucleotide receptors known as P2 receptors (P2Rs). P2Rs can further be subdivided into categories based on receptor type. P2YRs, of which 8 have been identified, are G protein-coupled receptors (121). P2XRs are cation-selective ion channels. Seven P2XR subunits have been cloned (P2X₁₋₇). From these 7 subunits, 6 P2XR homotrimer and 4 P2XR heterotrimer have been identified (120, 121). In addition to physiologic release mechanisms, ATP and other nucleotides are also released from cells following stress, cell membrane damage, and cell death (121, 122). The massive release of these nucleotides serves as a potent DAMP, and signaling through P2Rs is known to be important in initiation of inflammatory processes including cytokine release, as well as immune cell chemotaxis and activation (121, 122). Over time, the nucleotides are further broken down by cell surface enzymes (ectonucleotidases) to adenosine, which binds P1 receptors and elicits an overall anti-inflammatory response (123, 124).

Once ATP binds, P2X channels open, preferentially allowing sodium (Na⁺), potassium, and calcium (Ca²⁺) ions to cross the plasma membrane, ultimately leading to depolarization of the cell (125). These receptors have been identified on
most cell types and have important signaling functions both under physiologic conditions and during inflammation. P2XRs are well-documented at both pre- and post- synaptic sites in the central and peripheral nervous system and play an important role in fast signal transduction, neurotransmitter release, activation of carotid body chemoreceptors, stretch receptor activation in the bladder, and pain sensation (126-128). P2XRs have also been found in many other systems, including the following: 1) the cardiovascular system, where they help in the regulation of blood pressure and clotting; 2) the respiratory system, where they assist in clearance of mucus by ciliated epithelium; 3) the kidney, where they are implicated in regulating autoregulation of renal blood flow and glomerular filtration; 4) and the gastrointestinal tract, where they aid in intestinal motility and in secretion of various products by exocrine glands (126).

P2XRs can mediate cell death through a variety of mechanisms. The efflux of K+ via P2XRs is considered an important activator of the NLRP3 inflammasome, inducing both IL-1β production and pyroptotic cell death (129). Influx of Ca2+ is also associated with induction of apoptosis via activation of caspases-3, -8, and -9 (130). P2XR activation can also cause colloido-osmotic lysis, independent of caspase activation (125, 131). The intracellular C-terminal tail of the P2X7R is longer than that of any other P2XR, which imbues the receptor with unique permeability characteristics. Brief interaction with ATP results in similar selective permeability to Na+, K+, and Ca2+. However, prolonged activation results in a loss of selectivity, allowing other molecules including chlorine and propidium iodide to cross the cell membrane and can be associated with cell lysis (125).
P2X₁R, P2X₄R, and P2X₇R have been identified on most immune cells, including monocytes/macrophages, microglia, neutrophils, B and T lymphocytes, and mast cells (126). The role of these receptors in immune function has been an area of increasing interest. As mentioned earlier, activation of P2XRs, and the subsequent K⁺ efflux, is often associated with NLRP3 activation and IL-1β secretion. In many cases, inhibition of P2XR, or P2X₇R specifically, completely blocks IL-1β secretion (129, 132, 133). Besides their role in NLRP3 activation and immune cell death, P2XRs are known to play an integral role in immune cell function via stimulation of pro-inflammatory cytokine release, upregulation of iCAM on endothelial cells to aid in recruitment and extravasation of inflammatory cells, increased phagocytic capacity of macrophages and neutrophils, and direction of chemotaxis (121, 132). In the context of a mouse cecal ligation and puncture (CLP) model of polymicrobial sepsis, serum ATP levels positively correlate with neutrophil recruitment. Furthermore, when suramin, a non-specific P2 inhibitor, was administered to mice prior to infection, fewer neutrophils were identified in the blood 4 hours post-infection, indicating a defect in neutrophil recruitment (134). As early neutrophil recruitment has been found to be involved in rapid bacterial clearance and protection against CLP-induced sepsis (135), P2R-dependent neutrophil recruitment could serve as an important mechanism for protection during polymicrobial sepsis.

P2X₇R is by far the most well-characterized P2XR in the context of infection. During CLP-induced sepsis P2X₇⁻/⁻ mice were shown to have significantly decreased survival times, increased bacterial loads, and increased inflammatory
cytokine production following infection (136). Furthermore, P2X7R signaling specifically on macrophages was important for decreasing inflammatory cytokine production in vivo, an effect which was attributed to the capacity of P2X7R to improve bacterial killing by macrophages (136). Alternatively, P2X7R-induced VCAM-1 shedding has been shown to be a potent neutrophil chemoattractant in the context of non-infectious acute lung injury leading to increased tissue damage (137), suggesting that in some cases, P2XR inhibition may protect from pathologic inflammation.

The role of P2X4R and P2X1R in sepsis has also been evaluated. One study found P2X4R to be protective in a CLP model of sepsis and determined that P2X4R augmented bacterial killing via increased ROS production (138). Interestingly, following LPS challenge, P2X1R was protective during endotoxemia, which was attributed to dampened neutrophil activation, and reduced release of ROS (139), suggesting that activation of different P2XRs may have differential effects. However, in a model of LPS-induced lethal endotoxemia, P2X1−/− mice demonstrated reduced mortality and decreased tissue pathology, which was attributed to reduced extravasation of neutrophils into tissues (140). Overall, conflicting results between studies suggest that many factors, including tissue, cell type, P2XR subtype, and concentration and duration of ATP in the extracellular space, impact the role of P2XR signaling during infection, and further studies are needed to help define these conditions.

P2XRs are expressed on both immune and non-immune cells of the brain, and are known to be extremely important to neurologic function (120, 126). As
NLRP3 activation and IL-1β secretion have previously been associated with neurologic dysfunction following sepsis and meningitis (107, 108), and P2XR activation is frequently associated with NLRP3 and IL-1β, a logical extension would be to conclude that P2XRs could have a similar detrimental effect on the brain during infection. Interestingly, one study found that while inhibition of P2XR activation significantly decreased IL-1β and IL-6 secretion by macrophages following infection with *S. pneumoniae* *in vitro*, P2XR inhibition did not affect clinical scores, bacterial loads, or intracranial pressure following intracisternal infection with *S. pneumoniae* (141). This was attributed to decreased expression of P2XRs following infection, as well as decreased ATP in the CSF, which may have been due to rapid breakdown by macrophage- or neutrophil-derived ectonucleotidases (141). However, P2XR activity has also been associated with sepsis-induced neurologic dysfunction (142, 143). Following CLP-induced sepsis, mice had significantly increased oxidative damage in the cerebral cortex and hippocampus. This damage was significantly decreased in the hippocampus of P2X<sup>7</sup>−/− mice (142). P2X<sup>7</sup>−/− mice also had significantly reduced IL-1β and IL-6 in the hippocampus and cerebral cortex following CLP, indicating a diminished inflammatory response. The same study found that mice lacking CD39, an ectonucleotidase which converts ATP to adenosine monophosphate, had significantly increased IL-1β and IL-6 in the brain, but no difference in cytokine secretion into the blood, suggesting a specific role for CD39 in limiting P2XR activation in the brain during sepsis (142). Another study found that inhibition of P2X<sub>7</sub> following CLP leads to decreased ICAM-1 expression and leukocyte
adhesion in the brain vasculature, as well as decreased microglial cell activation and reduced brain damage (143), again suggesting an important role of P2XRs in the pathogenesis of septic encephalopathy. However, P2X7R-dependent IL-1β secretion has also been demonstrated to be important in normal memory development (144), suggesting that a delicate balance exists between physiologic and pathologic roles of P2XRs.

P2XR in neonatal immunity

To date, very little has been done to assess the function of P2XR signaling in neonates during infection or inflammatory conditions. P2X7 expression has been measured in macrophages from term and pre-term neonates and was found to be similar to expression in macrophages from adults (109). It is known that, compared to adult blood, neonatal blood contains higher levels of the ectonucleotidases required to break down ATP to the anti-inflammatory mediator adenosine and lower concentrations of the enzyme required to break adenosine down to its inert byproduct, inosine (145). This net increase in adenosine may be important for dampening potentially damaging inflammation, but could also inhibit pathogen clearance in the case of neonatal infection. One study assessed the effect of P2XR inhibition on survival of neonatal rats challenged with LPS. They also maintained surviving animals into adulthood to assess long-term alterations to anxiety behavior and pain sensitivity, as well as continued evidence of oxidative stress. Interestingly, P2XR inhibition significantly decreased mortality rates in LPS-treated pups. While P2XR inhibition did not significantly impact anxiety or pain sensitivity, adult mice that had received the P2XR inhibitor during the neonatal LPS challenge
produced lower levels of superoxide anion in the dentate gyrus, indicating decreased oxidative stress and a possible role of P2XRs in ongoing neurologic damage following infection (146). However, more research is needed to understand the role of these receptors during neonatal infection.

Role of P2XR in UPEC infection

HlyA is able to induce P2XR-dependent lysis of THP-1 cells and primary human monocytes, which is significantly reduced by inhibition of either P2X1R, P2X4R, or P2X7R (147, 148). As HlyA is an important virulence factor on UPEC isolates, it is not surprising that the role of P2XRs in UPEC infection has been assessed in multiple studies. Inhibition of P2X7R in a mouse model of pyelonephritis had no effect on early recruitment of neutrophils or on control of acute infection, but did lead to a significant decrease in renal fibrosis 14 days post-infection (149). Furthermore, P2X7−/− mice had fewer macrophages infiltrating the cortex of the kidney and diminished neutrophil clearance (149). Interestingly, it has recently been shown that P2X1R, P2X4R, and P2X7R may have different roles in the context of UPEC infection (150). Following intravenous (i.v.) infection with the HylA+ UPEC strain ARD6, P2X7−/− had significantly increased bacterial loads, increased mortality, elevated cytokine production, and more severe hemolysis than wildtype controls. Similarly, P2X4−/− mice had elevated hemolysis and cytokine production compared to wildtypes, although P2X4-deficiency did not have a significant effect on survival. Alternatively, P2X1−/− mice had significantly lower cytokine levels and reduced hemolysis in the blood following ARD6 infection, although this did not translate to a difference in overall survival (150). Together,
these data indicating a possible protective role of both P2X7R and P2X4R and a potentially detrimental effect of P2X1R activation in urosepsis.

**Purpose and experimental approach**

Although NMEC is one of the leading causes of sepsis and meningitis in newborn infants (22, 23), relatively little has been done to assess the role of innate immune signaling in the pathogenesis of infection, and even less has been done to determine how differences in neonatal immunity contribute to these infections. Therefore, we first focus on exploring the role of inflammasomes, an innate immune pathway known to be downregulated in newborn infants, in the pathogenesis of NMEC infection. Another challenge to developing treatments for NMEC infection is the variability in virulence factor expression between strains (20, 38). While many potential virulence factors have been identified in subsets of NMEC isolates, very few have been systematically assessed to determine their role in the pathogenesis of NMEC infection. Here we explore the role of one of these virulence factors, HlyA, focusing on how its ability to activate purinergic receptors potentially impacts the outcome of infection.
CHAPTER II

Materials and Methods

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 plates at 37°C and then cultured in Luria-Bertani broth for approximately 18 hours 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.

A description of the strains can be found in Table 1. Phylotyping and O-group serotyping was performed at the University of Georgia. To identify lactose-fermenting (Lac+) strains, bacteria were plated on MacConkey agar and incubated at 37 °C. Hemolytic ability was assessed by incubating bacteria on blood agar for 24-48 hours. NMEC strains were genotyped for cnf1 and hlyA using primers described by Wijetunge et. al. (20). Expression of nlp1 was used as a positive control and was identified in all strains.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Phylotype</th>
<th>O type</th>
<th>K type</th>
<th>Lac</th>
<th>Hemolysis</th>
<th>CNF-1</th>
<th>HlyA</th>
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<tr>
<td>NMEC RS218</td>
<td>B2</td>
<td>18</td>
<td>1</td>
<td>+</td>
<td>β</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NMEC O18</td>
<td>B2</td>
<td>18</td>
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<tr>
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<td>B2</td>
<td>18</td>
<td>1</td>
<td>+</td>
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<td>B2</td>
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<td>1</td>
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<td>γ</td>
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<tr>
<td>NMEC 83</td>
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<td>+</td>
<td>γ</td>
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<tr>
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<td>+</td>
<td>β</td>
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**Table 1. Genotype and phenotype of NMEC strains**

NMEC strains donated by Dr. Nolan and Dr. Logue at the University of Georgia. Phylotyping, O-group and K-group provided by collaborators at the University of Georgia. To assess lactose fermentation (Lac), bacteria was plated on MacConkey agar for 24 hours. Pink colonies were identified as lactose fermenters while colorless colonies were identified as non-lactose fermenters. To assess hemolysis, bacteria was plated on blood agar for 24 hours. Strains were described as β-hemolytic if a clear region formed around colonies, and γ-hemolytic if no hemolysis was appreciated. Strains were genotyped for CNF-1 and α-hemolysis using primers from Wijetunge et al. (20).
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-hour 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\(^{-/-}\), Caspase 1/11\(^{-/-}\), IL-1R\(^{-/-}\), Caspase 11\(^{-/-}\) and NOS2\(^{-/-}\) breeder pairs were originally obtained from Jackson Laboratory. Caspase 8\(^{fl/fl}\)-LysMcre, and Casp8\(^{fl/fl}\)-LysMcre Casp1/11\(^{-/-}\) mice were provided by Dr. Deborah Anderson and Dr. Rachel Olson at the University of Missouri, and P2X\(_7\)^{-/-} mice were provided by Dr. Gary Weisman at the University of Missouri.

Macrophage generation

Peritoneal macrophages were elicited by administering thioglycolate intraperitoneally 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. 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, and 10% heat inactivated fetal bovine serum (FBS)], plated at 1x10\(^6\) cells/ mL and incubated at 37°C with 5% CO\(_2\) overnight to allow for adhesion to the plate prior to use in experiments (151).

Bone marrow-derived macrophages (BMDMs) were generated as previously described (152). In short, bone marrow was flushed from the femurs and tibias of 6-12-week-old mice and cultured in CM containing 30 ng/mL M-CSF.
Fresh media was added after 3 days, and adherent cells were collected at 6- or 7-days post-harvest. Cells were plated at $1 \times 10^6$ cells/mL and left to adhere overnight.

A microglial cell line derived from C57BL/6 mice (NR-9460) was obtained from BEI Resources. MyD88/Mal

- (NR-9904), TLR4

- (NR-9901) and TRIF

- (NR-15638) microglial cell lines on the same background were also obtained from BEI Resources. Cells were cultured in CM and washed and split every 2-4 days. Cells from passages 3-5 were used in experiments, at which time the cells were washed and adherent cells were collected into fresh CM using a cell scraper. Cells were then plated at $1 \times 10^6$ cells/mL and left to adhere overnight.

**In vitro infection, cytokine quantification and assessment of cell death**

Cells were washed with PBS and either CM or Opti-MEM was added prior to infection with *E. coli*. Treatment with cytochalasin-D (10 µM) (153, 154), potassium chloride (KCl; 50 mM) (155), glycine (10 mM) (156, 157), necrosulfonamide (20 µM) (158), necrostatin-1 (10 µM) (159), z-VAD (100 µM) (160) or BBG (100 µM) (161) was initiated at the time cells were washed, approximately 1 hour 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 hours post-infection, treated with penicillin-streptomycin and stored at -20°C until the time of analysis. For LPS/nigericin groups, 200ng/ml LPS was added to wells and 20 µM nigericin was added 4 hours post-LPS treatment. Supernatants were collected 1 hour following nigericin treatment and frozen until the time of analysis. Cell supernatants were assessed for cytokine production using ELISA kits: Mouse IL-1β (Invitrogen), Mouse IL-1α
(Invitrogen), and Mouse IL-6 (eBioscience) according to manufacturers’ instructions. IL-18 production was assessed using magnetic bead reagents (eBioscience) and the Luminex MagPix system according to manufacturer’s instructions.

For in vitro experiments assessing the relationship between nitric oxide (NO) and IL-1β, BMDMs were infected with NMEC RS218 at a MOI of 0.1 or 1. Cells were washed to remove extracellular bacteria at 3-hours post-infection and fresh CM containing 50 µg/ml gentamicin was added. 500 µM S-nitroso-N-acetyl-penicillamine (SNAP, Sigma) was added to some cells at the time of gentamicin treatment. Supernatants were collected 17-49 hours after the addition and gentamicin and stored at -20˚C prior to analysis of IL-1α, IL-1β and IL-6 and determination of nitric oxide production via the Griess reaction(162). Following collection of supernatants, the cells were washed with PBS and lysed with sterile ddH₂O. The cell lysate was diluted and plated on MacConkey agar to determine intracellular bacterial loads.

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

To assess uptake of bacteria and bacterial killing by macrophages, BMDMs were infected with NMEC RS218 or NMEC O18. One-hour post-infection, cells were washed with sterile PBS, and media replaced with CM containing 50 µg/mL
gentamicin. Cells were then washed and lysed 1.5-, 5-, or 24-hours post-infection. Lysates were plated on MacConkey agar to determine intracellular bacterial loads.

**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 incorporation (152). The following primers from Integrated DNA Technologies were used: IL-1β forward, 5’- ATCCCACCTTTTGACAGTGAT -3’ and reverse, 5’- ATCAGGACAGCCCGCTCAA -3’; and GAPDH forward, 5’- GTGGACCTGGCCTACAT -3’ and reverse, 5’- GGGTGCAGCGAACTTTATTG -3’.

**Adult mouse intracerebral infections**

6-12-week-old mice were anesthetized i.p. with 100 mg/kg ketamine and 10 mg/kg xylazine. For i.c. injections a 26s-gauge needle attached to a 5 µL Hamilton syringe was inserted through the skull to a depth of 3.0-3.5 mm. Needle placement was approximately ½ the distance between the eye and the ear, and just to the right of midline (87, 163). 100-500 CFU NMEC RS218, in 1 µL PBS was slowly instilled into the brain over 1 minute, and the needle was held in place for an additional 30 seconds prior to removal.
For survival studies, mice were monitored frequently following infection. Humane endpoints were determined based on a University of Missouri IACUC approved clinical scoring system which accounted for the mentation/activity level of the mice, posture/appearance, and manifestation of neurologic signs. Weights were also monitored at least once daily. However, we did not find weight loss to be associated with severity of disease, likely due to the acute nature of the infection, and therefore did not include weight loss as a cut off for euthanasia.

For assessment of bacterial loads in the brain, mice were euthanized 16-18 hours post-infection. The brain was removed and homogenized in 900 µL PBS. Serial 1:10 dilutions were plated on MacConkey agar to determine bacterial loads.

**Neonatal mouse infection**

Three-day-old mice were infected i.p. with 50-10000 NMEC RS218 or NMEC O18 as described in the figure legends. Except for data comparing bioactive IL-1 production (Figure 14), 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. 50 mg/kg sterile-filtered BBG (Sigma) in 25 µL PBS (146, 150, 164) or an equivalent volume of PBS were delivered i.p. approximately 2 hours prior to infection, and readministered 24 hours later for survival studies.

For survival studies, mice were monitored frequently following infection. Blood was collected 18 hours post-infection via the facial vein for confirmation of bacteremia. Mice were euthanized and counted as a mortality if they appeared unresponsive at the time of checks.
For assessment of bacterial loads and cytokine production at 6-, 12- or 18-hours 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 and blood samples were then spun down to remove cellular portions, and all samples were treated with antibiotics and frozen at -20°C for future use in the assessment of cytokine production. Peritoneal lavage samples were assessed for cytokine production using a multiplexed magnetic bead assay (eBioscience) and the Luminex MagPix system according to manufacturer's instructions.

**IL-1R blockade and cell depletions in neonatal mice**

For IL-1 receptor blocking experiments, a single dose of 40 mg/kg anti-IL-1R antibody (JAMA-147, BioXCell) or hamster IgG (Southern Biotechnology) was administered i.p. in conjunction with the inoculum. This dose was extrapolated from studies performed in adult mice which utilized a single dose of approximately 10-30 mg/kg JAMA-147 (165). JAMA-147 was detected via ELISA in both the brain and spleen of neonatal mice 18 hours after infection with NMEC RS218 (166).

To deplete neutrophils, mice were treated with 15 µg (approximately 5 mg/kg) with anti-Ly6G antibody (clone 1A8, Leinco) or rat IgG (Southern Biotechnology) i.p. on post-natal day 1-3 (88). Depletion was confirmed by flow cytometry 18 hours post-infection.
To deplete macrophages, mice were treated with a single dose of 15 mg/kg clodronate liposomes (Encapsula Nano Science) i.p. on post-natal day 2 (167). Depletion was confirmed by flow cytometry 18 hours post-infection.

**Bioactive IL-1 reporter cells**

HEK-Blue IL-1 reporter cells (Invivogen) were used to detect bioactive IL-1. This cell line stably expresses the mouse IL-1 receptor. Binding of active IL-1β and/or IL-1α to the IL-1R triggers a signaling cascade leading to production of secreted alkaline phosphatase (SEAP), which can subsequently be detected in supernatants using QUANTI-Blue reagent (Invivogen). Cells were initially cultured in a T25 flask with 6 ml growth media (DMEM, 4.5 g/L glucose, 10% heat inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL normacin). Cells were split 1:8 when they reached ~80% confluency (about every 3 days). After the second passage, 100 µg/mL hygromycin B gold, 200 µg/mL puromycin and 1µg/mL zeocin was added to the growth media. For experiments, cells were washed and suspended in test media (DMEM with 4.5 g/L glucose, 10% heat inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin) and 180 µL were added to the wells of a 96-well plate along with 20 µL of peritoneal lavage fluid from infected neonates. As these cells can also produce SEAP in response to TNF-α (168), all lavage samples were first incubated on a plate coated with anti-mouse TNF-α (eBioscience) before use. Recombinant IL-1β (eBioscience) was used to create a standard curve starting at 10 ng/mL. Reporter cells were incubated overnight at 37°C with 5% CO₂. Next, 10 µL supernatant from the reporter cells was added to 90 µL QUANTI-Blue solution in a 96-well plate and
incubated for 30 minutes at 37°C. Optical density was measured with a spectrophotometer at 650 nm.

**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 $P < 0.05$. Statistical significance of survival studies was determined using log rank analysis with significance set at $P < 0.05$. *In vitro* data was normally distributed, and therefore analyzed via the Student $t$-test (when comparing two groups), or via ANOVA followed by Tukey’s test (when comparing 3 or more groups) with significance set at $P < 0.05$ as described in the figure legends. All error bars represent the standard deviation of the sample mean.
CHAPTER III

Nitric Oxide Inhibits IL-1-Mediated Protection Against *Escherichia coli* K1-Induced Sepsis and Meningitis in a Murine Neonatal Model

ABSTRACT

Neonatal meningitis-associated *E. coli* (NMEC) is a leading cause of sepsis and meningitis in newborn infants. Neonates are known to have impaired inflammasome activation and IL-1 production. However, it is unknown what role this plays in the context of NMEC infection. Here we investigated the role of IL-1 signaling in the pathogenesis of NMEC infection. We found both IL-1β and IL-1α were secreted from macrophages and microglial cells in response to NMEC in a TLR4- and NLRP3- dependent manner. Intracerebral infection of adult mice indicated a protective role of IL-1 signaling during NMEC infection. However, IL-1R blockade in wildtype neonatal mice did not significantly alter bacterial loads in the blood or brain, and we therefore investigated whether protection conferred by IL-1 was age dependent. Neonates are known to have increased nitric oxide (NO) levels compared to adults, and we found NO inhibited the secretion of IL-1 by macrophages in response to NMEC. In contrast to our results in wild-type neonates, blockade of IL-1R in neonates lacking inducible nitric oxide synthase (iNOS) led to significantly increased bacterial loads in the blood and brain. These data indicate IL-1 signaling is protective during NMEC infection in neonates only
when iNOS is absent. Collectively, our findings suggest increased NO production by neonates inhibits IL-1 production, and that this suppresses the protective role of IL-1 signaling in response to NMEC infection. This may indicate a general mechanism for increased susceptibility of neonates to infection and could lead to new therapeutic strategies in the future.

INTRODUCTION

Neonatal meningitis-associated *Escherichia coli* is a distinct pathotype of ExPEC which causes a high degree of bacteremia leading to sepsis and meningitis in newborn infants (20, 21). NMEC is the second leading cause of bacterial meningitis in neonates, accounting for approximately 30% of cases (22, 45). Furthermore, NMEC is considered the leading cause of bacterial meningitis in premature and VLBW infants (22). Even when antibiotic treatment is successful, surviving infants are at risk of long-term neurologic sequelae. It is estimated as many as 30-50% of infants with a history of meningitis will have some degree of neurologic impairment throughout life, ranging from hearing loss, to learning disabilities to seizure disorders (41, 169). Additionally, the rise in multi-drug-resistant NMEC isolates is a growing concern. Recent studies have found approximately 70% of new NMEC isolates are ampicillin-resistant, and multi-drug-resistant isolates are also being identified (43, 44). This rise in antimicrobial resistance in conjunction with the continued high morbidity and neurologic impairment associated with these cases has precipitated a need for research into new therapeutics. Development of novel therapeutics requires an in depth
understanding of the role that host immune responses play in the pathogenesis of infection. To that end, we investigated the role of inflammasome-dependent IL-1 signaling in NMEC infection.

Inflammasomes are cytosolic signaling complexes which assemble and become activated in response to DAMPs and PAMPs (92). Once activated, inflammasomes cleave pro-caspase-1 to its functional form, caspase-1. Activated caspase-1 is then able to initiate pyroptosis, a form of programmed cell death, and activate both IL-18 and IL-1β (92).

IL-1β is a pleiotropic pro-inflammatory cytokine which binds the IL-1 receptor (IL-1R). IL-1β is not constitutively expressed in myeloid cells. Instead, pro-IL-1β is induced in response to signaling from pattern recognition receptors, such as TLRs, and must then be catalytically cleaved to its active form by caspase-1 following inflammasome activation (101). In contrast, IL-1α, another member of the IL-1 family which also binds to and activates IL-1R, is constitutively expressed in many cell types and can be released in an inflammasome-dependent or -independent manner (102, 170).

IL-1 signaling has previously been investigated in the pathogenesis of other forms of bacterial meningitis. IL-1α and IL-1β are both induced by Streptococcus pneumoniae, the causative agent of pneumococcal meningitis, and IL-1 signaling was found to be important for bacterial clearance and survival of 3-4 week old mice (105). Additionally, others have found IL-1R-deficient adult mice were significantly more susceptible to sepsis and meningitis caused by Streptococcus agalactiae, the leading cause of meningitis in neonates (104). However, the specific role of IL-
1 in NMEC infection, as well as its general role in neonatal infections, has not been fully explored.

Nitric oxide is a small bioactive molecule involved in a wide range of physiologic responses. NO production is mediated by three nitric oxide synthases. iNOS is upregulated in response to infection, and plays a variety of roles in immune function (171). However, the effect of iNOS activity on infection outcome varies depending on the pathogen and physiologic environment. It has previously been shown that in the context of *E. coli* K1 infection of neonatal mice, iNOS deficiency improved bacterial clearance and improved survival (81). It is known that NO levels are elevated in neonates (78, 80), and that NO has the potential to inhibit IL-1 production via s-nitrosylation of NLRP3 (152, 172). Therefore, we assessed how NO regulates IL-1 production in response to NMEC and how this affects the outcome of infection.

**RESULTS**

**NMEC RS218 infection induces IL-1β and IL-1α release**

To determine whether NMEC RS218 activates inflammasomes, peritoneal macrophages were infected with NMEC RS218, and supernatants were assessed for IL-1β. Interestingly, we found NMEC RS218 induced a robust IL-1β response indicative of inflammasome activation (Fig. 1A). NMEC RS218 infection also led to significant induction of IL-18, further suggesting inflammasome activation (Fig. 1C). Both IL-1β and IL-1α signal through IL-1R, and we found that IL-1α was also secreted by peritoneal macrophages upon NMEC RS218 infection (Fig. 1B). In
addition, we found that bone marrow-derived macrophages (BMDMs) secreted significant levels of both IL-1β and IL-1α in response to NMEC RS218 (Fig. 2A and B), indicating IL-1 induction in response to NMEC RS218 is not unique to peritoneal macrophages. Due to the proclivity of NMEC to cause meningitis, we infected microglial cells, the resident macrophage-like cells of the brain (173). We again found NMEC RS218 infection induced a significant IL-1 response as well as secretion of IL-18 (Fig. 3A-C). Taken together, these data suggest NMEC activates inflammasomes.

**IL-1β and IL-1α are induced in a TLR4 and MyD88 dependent manner**

We next explored the signaling pathways involved in the induction of IL-1 during NMEC RS218 infection. It is well known that LPS from gram-negative organisms such as *E. coli* is recognized by TLR4 (92, 170). By infecting wildtype and TLR4−/− microglial cells with NMEC RS218, we determined that IL-1β and IL-1α secretion is entirely dependent on TLR4 signaling (Fig. 4A and B). We also found IL-6 secretion was markedly attenuated in TLR4−/− microglia, indicating a general defect in the inflammatory response of TLR4−/− cells to NMEC RS218 (Fig. 4C).

TLR4 can signal through both the adaptor proteins MyD88 and TRIF (174). To determine which of these signaling proteins is responsible for IL-1 secretion during NMEC RS218 infection, we infected MyD88/Mal−/− and TRIF−/− microglia. Microglia lacking MyD88/Mal did not produce IL-1β in response to NMEC RS218 (Fig. 4D). MyD88/Mal was also required for production of IL-1α and IL-6 in response to NMEC RS218 (Fig. 4E and F). In contrast, TRIF−/− microglial cells
Figure 1. NMEC RS218 elicits secretion of IL-1β, IL-1α and IL-18 from peritoneal macrophages.
Wildtype thioglycolate-elicited peritoneal macrophages were infected with NMEC RS218 at a MOI of 0.1 or 1. Supernatants were collected 5 hours post-infection and IL-1β (A), IL-1α (B) and IL-18 (C) were quantified. Data are representative of 2 or 3 independent experiments each with 3 wells per group. Means with the same letter are not statistically different from each other with $P < 0.05$. The error bars represent S.D. of the mean.
Figure 2. NMEC RS218 elicits secretion of IL-1β and IL-1α from bone marrow-derived macrophages.
Bone marrow-derived macrophages were infected with NMEC RS218 at a MOI of 0.1 or 1. Supernatants were collected 5 hours post-infection, and IL-1β (A) and IL-1α (B) were quantified by ELISA. Data are representative of 2 or 3 independent experiments each with 3 wells per group. Means with the same letter are not statistically different from each other with \( P < 0.05 \). The error bars represent S.D. of the mean.
Figure 3. NMEC RS218 elicits secretion of IL-1β, IL-1α and IL-18 from microglial cells.

Cell of the mouse microglial cell line NR-9460 were infected with NMEC RS218 at a MOI of 0.1 or 1. Supernatants were collected 5 hours post-infection and IL-1β (A), IL-1α (B) and IL-18 (C) were quantified. Data are representative of 2 or 3 independent experiments, each with 3 wells per group. Means with the same letter are not statistically different from each other with $P < 0.05$. The error bars represent S.D. of the mean.
produced similar levels of IL-1α as wildtype cells (Fig. 5B), and while IL-1β levels were somewhat decreased in TRIF<sup>−/−</sup> cells, the difference was not statistically significant (Fig. 5A). These data indicate that MyD88/Mal rather than TRIF is required for IL-1 secretion in response to NMEC RS218.

To confirm these observations were not unique to microglial cells, we infected wildtype and MyD88<sup>−/−</sup> peritoneal macrophages. We found that like microglia, peritoneal macrophages secrete IL-1β, IL-1α and IL-6 in a MyD88-dependent manner (Fig. 6A-C). Taken together, these data indicate the IL-1 response to NMEC RS218 is TLR4 and MyD88 dependent.

**IL-1β and IL-1α secretion is NLRP3 and Caspase-1 dependent**

Since activation and release of IL-1β is typically dependent on caspase-1 and inflammasome activation, we sought to determine whether inflammasomes are responsible for secretion of IL-1β during NMEC infection. The NLRP3 inflammasome can become activated in response to potassium efflux following cellular damage (97). It has previously been shown that UPEC induce IL-1β via activation of NLRP3. Furthermore, NLRP3 activation was generally associated with UPEC expression of the pore-forming toxin HlyA (113, 175).

NMEC RS218 possesses a hlyA gene that shares over 98% sequence identity with hlyA from UPEC (20, 176). Therefore, we suspected the NLRP3 inflammasome may be involved in the release of IL-1 during NMEC infection. To test this, we infected peritoneal macrophages from wildtype, NLRP3<sup>−/−</sup>, and Caspase1/11<sup>−/−</sup> mice with NMEC RS218 and analyzed the supernatant for IL-1β. Indeed, we found Caspase1/11<sup>−/−</sup> cells were unable to release IL-1β in response to
Figure 4. IL-1 and IL-6 production by microglial cells in response to NMEC RS218 is TLR4 and MyD88/Mal dependent.

Wildtype (WT), TLR4\(^{-/-}\) (A-C) or MyD88/Mal\(^{-/-}\) (D-F) microglial cells were infected with NMEC RS218. Supernatants were collected 5 hours post-infection and analyzed for IL-1\(\beta\) (A and D), IL-1\(\alpha\) (B and E) and IL-6 (C and F). Data are representative of 2 or 3 independent experiments, each with 3 wells per group. *, \(P < 0.05\). The error bars represent S.D. of the mean.
Figure 5. TRIF signaling does not significantly contribute to IL-1 production in response to NMEC RS218 infection

Wildtype (WT) and TRIF-/- microglial cells were infected with NMEC RS218. Supernatants were collected 5 hours post-infection and analyzed for IL-1β (A) and IL-1α (B). Data are representative of 2 or 3 independent experiments, each with 3 wells per group. N.S. = not significant with significance set at \( P < 0.05 \). The error bars represent S.D. of the mean.
Figure 6. IL-1 and IL-6 secretion by peritoneal macrophages is dependent on MyD88.
Wildtype (WT) and MyD88−/− thioglycolate-elicited peritoneal macrophages were infected with NMEC RS218. Supernatants were collected 5 hours post-infection and analyzed for IL-1β (A), IL-1α (B), and IL-6 (C). Data are representative of 2 or 3 independent experiments, each with 3 wells per group. *, P < 0.05. The error bars represent S.D. of the mean.
infection (Fig. 7B). Additionally, we found NLRP3−/− cells did not secrete IL-1β (Fig. 7B), indicating IL-1β release in response to NMEC RS218 infection is dependent on activation of NLRP3. While virtually no IL-1β was secreted by NLRP3 deficient cells, IL-1β mRNA expression was induced nearly 1000-fold in NLRP3−/− macrophages following infection, indicating NLRP3 is not essential for IL-1β priming (Fig. 7A).

The precursor of IL-1α is constitutively expressed in many cell types and can be released from cells in an inflammasome-dependent or independent manner. The IL-1α precursor is biologically active, unlike pro-IL-1β, and may function as an alarmin in response to cellular damage (102, 170). Interestingly, when we infected wildtype, NLRP3−/−, and Caspase1/11−/− macrophages, we found IL-1α secretion was NLRP3- dependent (Fig. 7C). Therefore, it appears the release of both IL-1α and IL-1β is dependent on NLRP3 activation following NMEC RS218 infection.

While IL-1α can be released passively following cell death, both IL-1α and IL-1β may also be released via inflammasome-dependent GSDMD pores in the absence of cell death (102, 177). We found that the osmoprotectant glycine inhibited cell death without affecting IL-1α or IL-1β release (166). These data indicate cell death is not responsible for IL-1α or IL-1β release in response to NMEC RS218. To ensure impaired IL-1 release was not due to a general effect on the inflammatory response, we also analyzed supernatants for IL-6, a non-inflammasome-dependent inflammatory cytokine and found secretion of IL-6 was reduced ~50% but was not completely dependent on caspase1/11 and NLRP3 (Fig. 7D).
Figure 7. IL-1β and IL-1α production by peritoneal macrophages in response to NMEC RS218 is NLRP3-inflammasome dependent.

(A) NLRP3−/− peritoneal macrophages were infected with NMEC RS218 (MOI 0.1). Cell lysates were collected 5 hours post-infection, RNA was purified and qRT-PCR was performed. IL-1β expression was calculated as fold change compared to mock infected NLRP3 deficient cells $2^{\Delta\Delta Ct}$ (B-D) Wildtype (WT), NLRP3−/− and Caspase 1/11−/− were infected with NMEC RS218. Supernatants were collected 5 hours post-infection and analyzed for IL-1β (B), IL-1α (C) and IL-6 (D). Data in (A and D) are representative of 1 experiment while data in (B and C) are representative of 2 or 3 independent experiments each with 3 wells per group. *, $P < 0.05$. Means with the same letter are not statistically different from each other. The error bars represent S.D. of the mean.
To determine whether NLRP3 activation by NMEC RS218 is conserved across cell types, we infected microglial cells treated with MCC950, an inhibitor of NLRP3 dependent caspase-1 activation (178). NLRP3 inhibition blocked the ability of microglial cells to secrete IL-1β and IL-1α (Fig. 8A and B), but not IL-6 (Fig. 8C). Taken together, this indicates the secretion of both IL-1β and IL-1α is dependent on NLRP3 activation in response to NMEC RS218 infection.

Canonically, NLRP3 is activated in response to cell membrane disruption and K+ efflux. This cell membrane disruption may be caused directly by the bacterium or may be secondary to cell processes such as the activation of caspase-11 (92). Caspase-11 recognizes cytosolic LPS and robustly activates GSDMD. The subsequently formed GSDMD pores allow for K+ to leave the cell, therefore leading to secondary activation of NLRP3 (92). To determine the relative role of caspase 1 and 11 in the activation and release of IL-1 during NMEC RS218 infection, we compared IL-1 production from wildtype, caspase11−/− and caspase1/11−/− peritoneal macrophages. Interestingly, we found that, unlike caspase1/11−/− cells, macrophages lacking only caspase-11 were able to respond to infection by secreting both IL-1α and IL-1β (Fig. 9A and B). This indicates that caspase-1 but not caspase-11 is required for secretion of IL-1 in response to NMEC RS218.

**IL-1 signaling is protective during NMEC RS218 infection of adult mice**

IL-1 signaling can have both protective and deleterious effects in the context of infection depending on the infectious agent and the physiologic environment (179). To determine the role of IL-1 signaling during *in vivo* NMEC infection, we
Figure 8. IL-1β and IL-1α production by microglial in response to NMEC RS218 is NLRP3-inflammasome dependent.

Wildtype (WT) microglial cells were treated with PBS or the NLRP3 inhibitor MCC950 and infected with NMEC RS218. Supernatants were collected 5 hours post-infection and analyzed for IL-1β (A), IL-1α (B) and IL-6 (C). Data are representative of 2 or 3 independent experiments each with 3 wells per group. *, P < 0.05. NS = Not significant. The error bars represent S.D. of the mean.
Figure 9. IL-1β and IL-1α production by peritoneal macrophages requires caspase-1, but not caspase-11.

Wildtype (WT), Caspase 1/11−/−, and Caspase 11−/− peritoneal macrophages were infected with NMEC RS218 and supernatants were collected 5 hours post-infection and analyzed for IL-1β (A) and IL-1α (B). Data are representative of 2 or 3 independent experiments each with 3 wells per group. Means with the same letter are not statistically different from each other. The error bars represent S.D. of the mean.
infected adult wildtype and IL-1R$^{-/-}$ mice with NMEC RS218 using a previously described model of i.c. infection (87, 163) and monitored these animals for survival. Interestingly, we found that IL-1R$^{-/-}$ mice had significantly decreased survival compared to wildtype mice (Fig. 10A). Additionally, bacterial loads in the brains of IL-1R$^{-/-}$ mice were ~10 fold higher than in brains from control animals (Fig. 10B). Together these data indicate a protective role for IL-1 signaling during NMEC RS218 infection and suggest a role for IL-1 in bacterial clearance.

**Nitric oxide inhibits IL-1 mediated protection against NMEC RS218 in neonates**

Because NMEC is generally associated with early onset infection of human neonates, we next asked whether IL-1 signaling was protective in a neonatal model of infection. Three-day-old wildtype mice were infected i.p. with NMEC RS218 and simultaneously treated with an IL-1R blocking antibody or an isotype control. Interestingly, in contrast to what we observed in adult mice, blockade of IL-1R had no significant effect on bacterial loads in the brain or blood of infected pups 18 hours post-infection (Fig. 11A and B). Together with the adult infections, these data suggest a potential difference in the role of IL-1 signaling during adult and neonatal NMEC RS218 infection. While it is possible that differences in the route of infection (intracerebral vs. systemic) contributed to the apparent altered role of IL-1 in the adult and neonatal models, differences in neonatal immune function, and specifically in neonatal inflammasome function, could also contribute to a differential response. Human cord blood monocytes have impaired NLRP3 activation and decreased IL-1β secretion in response to LPS compared to adult
Figure 10. IL-1 signaling is protective during intracerebral NMEC RS218 infection of adult mice.
6-12-week-old wildtype (WT) and IL-1R⁻/⁻ mice were infected intracerebrally with 100-500 CFUs of NMEC RS218. In (A) mice were monitored for survival (n = 10). In (B) mice were euthanized at 16-18 hours post-infection and brains were homogenized to determine bacterial loads (n = 15-21). Data are combined from 2 or 3 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at P < 0.05.
Figure 11. IL-1R blockade does not enhance susceptibility to NMEC RS218 infection in neonates.

3-day-old wildtype mice were infected i.p. with ~1x10⁴ CFU NMEC RS218. Mice also received anti-IL-1R antibody or hamster IgG (isotype) at the time of infection. Neonates were euthanized 18 hours post-infection, and brain (A) and blood (B) were plated on MacConkey agar to determine bacterial loads. n = 12-16 per group. Data are combined from 4 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. NS = not significant with significance set at P = 0.05.
blood monocytes (109, 110). However, the underlying cause of these differences have not been fully explored. Neonates also express higher levels of NO, which can inhibit NLRP3 activation (152, 172). This could explain the impaired IL-1 response in our neonatal model.

To determine whether NO can inhibit IL-1 secretion in response to NMEC RS218, BMDMs were treated with the NO donor SNAP. Because NO can have direct bactericidal effects, macrophages were treated with SNAP only after infection was established. As expected, the addition of SNAP to NMEC RS218 infected cells significantly increased the concentration of NO within supernatants (Fig. 12A). Under these conditions, we found that bacterial levels were similar between control and SNAP-treated cells (Fig. 12B). However, SNAP-treated macrophages secreted ~50 to 90% less IL-1β and IL-1α (Fig. 12C and D). While SNAP markedly inhibited IL-1β secretion by BMDMs, IL-1β mRNA expression was lowered, but still robustly induced in infected cells treated with SNAP (Fig. 12E). SNAP-treated cells also produced similar amounts of IL-6, suggesting that decreased IL-1 secretion is not due to a general inhibition of inflammatory processes (Fig. 12F).

To further assess the link between NO production and IL-1 secretion, we infected wildtype and inducible nitric oxide synthase deficient (NOS2−/−) BMDMs with NMEC RS218. We initially found significant levels of NO in wells containing wildtype or NOS2−/− macrophages, which we subsequently found was due to NO production by extracellular NMEC RS218 (data not shown). We therefore optimized our conditions such that NO levels were lower in wells containing
Figure 12. Nitric oxide inhibits the secretion of IL-1 in response to NMEC RS218 infection.

Wildtype BMDMs were infected with NMEC RS218. After three hours, media was replaced with CM containing 50 µg/mL gentamicin with or without 500 µM SNAP. Supernatants were collected 20 hours post-infection and analyzed for NO production (A), IL-1β (C), IL-1α (D), and IL-6 (E). In (B), cell lysates were diluted and plated on MacConkey agar to enumerate intracellular bacteria. In (E), cells were lysed for RNA purification and subsequent qRT-PCR. IL-1β expression was calculated as fold change compared to mock infected cells 2^(ΔΔCt). Data from (A-D, and F) are representative of 2 or 3 independent experiments, each with 3 wells per group. Data from (E) are from one experiment with 3 wells per group. Means with the same letter are not statistically different from each other. NS = not significant. * P < 0.05. The error bars represent S.D. of the mean.
NOS2\(^{-/-}\) BMDMs than wells containing wildtype BMDMs during infection. The cells were washed and treated with gentamicin 3 hours post-infection to remove extracellular bacteria, and supernatants were collected 49 hours after addition of gentamicin. Under these conditions NOS2\(^{-/-}\) wells had significantly decreased NO compared to wildtype wells (Fig. 13A). Interestingly, under these optimized conditions, we found NOS2\(^{-/-}\) cells secreted up to 80% more IL-1\(\beta\) and IL-1\(\alpha\) than WT cells (Fig. 13B and C). In contrast, induction of IL-1\(\beta\) mRNA by RS218 was similar in WT and NOS2\(^{-/-}\) cells (Fig. 13D), further supporting that iNOS inhibits IL-1 secretion rather than priming in response to NMEC infection.

It has previously been shown that iNOS inhibition is protective during infection with a rifampicin resistant derivative of NMEC RS218 in neonatal mice (81). As we found NO inhibits IL-1 production \textit{in vitro} (Fig. 12 and 13), we sought to determine whether iNOS suppresses IL-1 in neonates \textit{in vivo}. At 12 hours post-infection, we found NOS2\(^{-/-}\) neonates had significantly increased bioactive IL-1 levels in their peritoneum relative to WT pups despite similar bacterial loads (Fig. 14A and B). To determine whether this difference in IL-1 secretion contributes to the protective effect of iNOS inhibition in neonatal mice, three-day old NOS2\(^{-/-}\) mice were infected with NMEC RS218 and simultaneously treated with either IL-1R blocking antibody or an isotype control. Interestingly, we found that in the absence of iNOS, bacterial loads in the brain, blood, peritoneum, and spleen were \(~10\) to \(1,000\)-fold higher in pups treated with IL-1R blocking antibody compared to IgG-treated pups (Fig. 15A-D). These data indicate that, in contrast to what we
Figure 13. NOS2\textsuperscript{-/-} macrophages secrete significantly more IL-1 despite similar mRNA induction.

Wildtype (WT) and NOS2\textsuperscript{-/-} BMDMs were infected with NMEC RS218. After three hours, media was replaced with CM containing 50 μg/mL gentamicin. Supernatants were collected 49 hours after the addition of gentamicin and analyzed for NO (A), IL-1\textbeta (B) and IL-1\textalpha (C). Data are representative of 2 or 3 independent experiments, each with 3-12 wells per group. In (D), cells were lysed for RNA purification and subsequent qRT-PCR. IL-1\textbeta expression was calculated as fold change compared to mock infected WT or NOS2\textsuperscript{-/-} cells respectively $2^{\Delta\Delta Ct}$. *, $P < 0.05$. N.S. = not significant. The error bars represent S.D. of the mean.
Figure 14. Bioactive IL-1 levels are higher in NOS2<sup>-/-</sup> neonates compared to wildtype neonates following NMEC infection.

3-day-old wildtype (WT) and NOS2<sup>-/-</sup> mice (n = 12-14) were infected intraperitoneally with 1x10<sup>4</sup> CFU of NMEC RS218. Mice were euthanized 12 hours post infection and the peritoneum was washed with 200 µL sterile PBS. Serial dilutions of lavage fluid were plated on MacConkey agar to determine bacterial loads (A). The remaining sample was centrifuged to remove cellular debris, depleted of TNF-α and plated along with HEK-Blue IL-1 reporter cells to determine levels of bioactive IL-1 (B). Data are combined from 4 independent infections (WT and NOS2<sup>-/-</sup> pups were infected on different days). The solid lines represent the sample median. The dashed line represents the limit of detection. Significance was set at P < 0.05.
Figure 15. IL-1 signaling is protective during neonatal NMEC RS218 infection in the absence of iNOS.

3-day-old NOS2<sup>−/−</sup> mice were infected with ~1x10<sup>4</sup> CFU NMEC RS218 i.p. Mice also received anti-IL-1R antibody or hamster IgG (isotype). Neonates were euthanized 18 hours post-infection, and brain homogenate (A), blood (B), peritoneal lavage (C), and spleen homogenate (D) were plated on MacConkey agar to determine bacterial loads. n = 11-13 per group. Data combined from 3 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at P < 0.05.
observed during infection of wildtype neonates, IL-1 signaling is protective in the absence of iNOS.

**DISCUSSION**

Although NMEC remains the leading cause of bacterial meningitis in premature and VLBW infants, relatively little research has been done to understand the role of immune mechanisms in the pathogenesis of NMEC infection. Here we investigated the role of IL-1 signaling in a murine model of *E. coli* K1-induced meningitis and sepsis. We found NMEC RS218 induces secretion of both IL-1β and IL-1α (Fig. 1-3). This secretion was found to be dependent on TLR4 signaling through the MyD88/Mal signaling pathway (Fig. 4-6). Furthermore, we found induction of both IL-1α and IL-1β is dependent on NLRP3 and caspase-1 (Fig. 7-9).

With the knowledge that NMEC RS218 could induce an IL-1 response from macrophages and microglial cells, we next investigated the role this response plays in the pathogenesis of infection *in vivo*. We found IL-1 signaling to be protective in adult mice following i.c. infection with NMEC RS218 (Fig. 10). It has previously been reported that adult mice lacking MyD88 were more susceptible to *E. coli* K1 i.c. infection, while TRIF−/− mice had similar susceptibility to wildtype mice (87). MyD88 is known to be important for signal transduction downstream of the IL-1 receptor (180, 181). Additionally, we found MyD88 was required for IL-1 release in response to NMEC RS218 (Fig. 4 and 6). Therefore, it is possible that the protective role of MyD88 during NMEC RS218 infection is related to its role in
the secretion of IL-1 and/or in the IL-1 signaling cascade. Furthermore, it has also been shown that depletion of neutrophils alone, or in combination with monocytes, leads to increased bacterial loads in adult mice infected i.c. with *E. coli* K1 (87). Activation of IL-1R via IL-1α and IL-1β is known to initiate many downstream signaling cascades. In particular, IL-1 signaling is known to play an important role in neutrophil recruitment in numerous infection models (182, 183). Therefore, it is possible the protective effect of IL-1 signaling is, at least in part, due to neutrophil recruitment to the site of infection.

Interestingly, when we infected neonatal mice with NMEC RS218, we found blockade of IL-1R had no effect on the level of bacteremia or bacterial loads in the brain (Fig. 11). However, a previous study found that administration of exogenous recombinant IL-1 to 10-12 day old C3H/HeJ mice prior to infection with an *E. coli* K1 strain resulted in improved bacterial clearance (184). These data suggested IL-1 can be protective in neonatal *E. coli* infection, and we then focused on investigating whether age-specific differences in IL-1 secretion and inflammasome function may be in part responsible for the susceptibility of neonates to NMEC RS218. It has previously been reported that neonates have decreased inflammasome activation compared to adults, culminating in decreased IL-1β secretion. Specifically, human cord blood monocytes display impaired NLRP3 activation and decreased IL-1β secretion (109). This difference was even more pronounced when cord blood monocytes were collected from pre-term infants (110). Decreased IL-1 secretion, and subsequent limited IL-1R activation could explain the lack of effect of IL-1R inhibition on the outcome of neonatal infection in
wildtype mice, and in part, the increased susceptibility of neonates to NMEC infection.

We and others have previously demonstrated that NO can decrease IL-1β release via inhibition of the NLRP3 inflammasome (152, 172), which we show here to be responsible for IL-1β and IL-1α secretion in response to NMEC RS218. Additionally, it has been reported that human neonates produce higher basal levels of NO compared to adults, and that neonatal microglial cells mount a more robust NO response to inflammatory stimuli despite decreased production of other inflammatory mediators (78, 80). If this is the case in the context of NMEC RS218 infection, it could explain why IL-1R does not have the same protective effect in neonates as in adult mice. To explore this possibility, we treated infected macrophages with the NO donor SNAP. Macrophages treated with SNAP produced decreased levels of IL-1α and IL-1β while maintaining IL-6 production in response to NMEC RS218 (Fig. 12), indicating NO can disrupt IL-1 release without suppressing the general inflammatory response. Infection of wildtype and NOS2−/− BMDMs further demonstrated that NO inhibits IL-1 secretion in response to NMEC RS218 (Fig. 13).

NO production is mediated by three distinct nitric oxide synthases. Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutively expressed and play roles in neuronal signaling and control of blood pressure respectively. In contrast, iNOS expression is induced in response to cellular damage and is therefore significantly upregulated during the course of many infections (171). Others have previously found that use of iNOS
deficient mice, or administration of the iNOS inhibitor, AGHS, improved the outcomes and led to decreased bacterial loads in NMEC infected neonatal mice (81). Importantly, the E. coli strain used in those studies was a rifampicin-resistant mutant of the NMEC RS218 strain used here. The protective effect of iNOS inhibition was suggested to be due to increased activation and phagocytic capacity of macrophages and neutrophils (81). As IL-1 can mediate monocyte, microglia and neutrophil activation (102, 185), we wanted to determine whether the protective effect of NO inhibition could be partly IL-1-dependent. Indeed, we found that NOS2−/− mice had significantly more bioactive IL-1 in their peritoneum following NMEC RS218 infection (Fig. 14) and that blocking IL-1R in NOS2−/− mice enhanced NMEC RS218 colonization of the brain, blood, and other tissues (Fig. 15). These data indicate IL-1 signaling is protective in the absence of iNOS and suggest that the protective effect of iNOS inhibition is in part dependent on IL-1 signaling. In addition, as wildtype and NOS2−/− pups were treated with the same dose of anti-IL-1R antibody, the absence of an effect of IL-1R blockade in wildtype mice was likely not due to inadequate antibody dosing.

While iNOS is deleterious to control of NMEC RS218 (81), in a murine model of infection with the uropathogenic E. coli strain UPEC 1177 (O1:K1:H7), it was found that NOS2−/− and wildtype mice had similar bacterial loads in the kidney and bladder (186). Interestingly, the study noted that UPEC 1177 is a hemolysin negative strain, and it has previously been shown that, while IL-1 secretion is protective during UPEC infection, its induction generally requires activation of NLRP3 by UPEC alpha hemolysin (113, 175, 187). Therefore, as UPEC 1177
would likely not induce a significant IL-1 response, the lack of iNOS function in UPEC 1177-infected mice would likely not alter the outcome of infection in an IL-1-dependent manner. Further studies using HlyA-expressing UPEC would be needed to determine whether iNOS inhibition of IL-1-mediated protection is a common pathophysiologic mechanism associated with ExPEC infection.

_E. coli_ is considered one of the leading causes of early-onset bacterial meningitis in human infants, second only to GBS. Similar to what we found during NMEC RS218 infection, GBS induces IL-1 secretion via activation of NLRP3 (188). Furthermore, in an adult mouse model of GBS infection, mice lacking IL-1R were found to have defects in neutrophil recruitment, and mice lacking either IL-1R or NLRP3 had significantly higher mortality compared to wildtype mice (104, 188). This coincides with our findings that NLRP3-dependent IL-1 is protective in an adult mouse model of NMEC RS218 infection (Fig. 7, 8 and 10). However, to our knowledge the role of IL-1 has not been assessed in a neonatal model of GBS infection. Therefore, it is possible that a similar mechanism of NLRP3 suppression by increased nitric oxide could be involved in the pathogenesis of neonatal GBS infection. It has been shown that i.c. infection of 11-day-old rats with GBS initiated significant upregulation of iNOS and significantly increased NO production in the meninges. Furthermore, treatment with AGHS led to increased bacterial loads, increased occurrence of seizures and increased neuronal damage (189). The increase in neuronal damage following iNOS inhibition was attributed to the vasodilative effect of NO preventing cerebral ischemia during infection (189). While this does not mean that NO does not regulate IL-1 secretion in GBS infection, it
does indicate that other protective effects of NO in the context of GBS infection could have a greater effect on disease progression than the potential downregulation of IL-1. Further research is needed to explore NO-dependent inhibition of NLRP3 and IL-1 production as a general mechanism of increased susceptibility to infection in newborn infants.

While NO is frequently associated with bactericidal effects and improved bacterial clearance, NO can also be associated with cytotoxicity and exacerbation of immunopathology. For this reason, there has been great interest in manipulating NO production, with particular emphasis on development of specific iNOS inhibitors allowing for control of excess induced NO without compromising NO production by constitutively expressed eNOS and nNOS which is required to maintain proper vascular permeability and neuronal signaling. While several specific iNOS inhibitors have been identified for use in animal models, none so far has translated to a viable treatment in human patients (171). Therefore this remains an important area of future study.

Taken together, the data presented here indicate that IL-1 is induced by NMEC in a TLR4 and MyD88 dependent manner, and requires NLRP3 for release from macrophages. This IL-1 production has the potential to be protective during NMEC infection, perhaps due to increased neutrophil migration, or increased bacterial phagocytosis and killing by macrophages and neutrophils. However, in neonates, which have increased levels of NO, iNOS activity inhibits the protective effects of IL-1 during NMEC infection. Our results also suggest that neonates are more at risk of developing sepsis and meningitis in part due to their elevated levels
of nitric oxide, decreased capacity to activate the NLRP3 inflammasome, and
decreased ability to mount an IL-1 response (78, 80, 109, 110). This risk is
potentially higher in premature infants, as they have previously been shown to
have even higher basal NO levels than full-term infants (78) and may explain why
this group is at the highest risk of *E. coli*-induced meningitis. NO-dependent
inhibition of inflammasome activation in neonates may also serve as a contributing
factor to the general increased susceptibility of neonates, and in particular, pre-
term infants, to a wide range of infections. Further study into the role of neonatal
NO production and its effects on inflammasome signaling may yield valuable
information leading to new and improved therapeutics for neonatal infections.
CHAPTER IV

P2XRs Mediate Protection Against Neonatal Sepsis and Meningitis Caused by Alpha-hemolysin-Expressing *E. coli* K1 in a Macrophage-Dependent Manner

ABSTRACT

Neonatal meningitis-associated *E. coli* are among the leading causes of bacterial meningitis and sepsis in newborn infants. Several virulence factors have been identified as common among many NMEC strains, and have even been shown to play an important role in development of bacteremia or meningitis. However, there is still significant variability in the virulence factor expression between NMEC isolates, and relatively little research has been done to assess the impact of many of these factors on immune cell activation and the outcome of infection. Here we investigate the role of bacterial strain-dependent P2XRs signaling on the outcome of infection in a neonatal sepsis model. We found that HlyA-expressing NMEC strains induced P2XR-dependent macrophage cell death *in vitro*, while HlyA⁻ strains did not induce a significant cell death response. This cell death was independent of IL-1β secretion, suggesting an uncoupling of P2XR and NLRP3 activation in the context of NMEC infection. *In vivo* inhibition of P2XRs was associated with increased mortality in HlyA⁺ NMEC strain infected neonatal mice, but had no effect on survival of HlyA⁻ NMEC infected neonates. Furthermore, neonatal mice depleted of macrophages prior to P2XR inhibition and infection with
the HlyA+ strain NMEC RS218 did not have significantly different bacterial loads from mice with intact P2XR signaling. Together, these data suggest that HlyA-dependent P2XR activation can mediate protection against NMEC infection, and requires macrophages. Future research should take into consideration the possible impact of various virulence factors when studying the immune response to NMEC infection.

INTRODUCTION

*E. coli* is a leading cause of sepsis in newborn infants, and the leading cause of meningitis in premature infants (21, 22). Neonatal meningitis-associated *E. coli* is a distinct pathotype of ExPEC associated with both sepsis and meningitis in infants (20, 38). 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 (190). Furthermore, a number of antibiotic resistant NMEC strains have recently been identified which may complicate treatment (43-49). Finally, even when antibiotic treatment is successful, infants that survive these infections are at a significantly increased risk of life-long neurologic sequelae ranging from learning disabilities to seizure disorders (22, 40, 41). These continued limitations precipitate the need for new therapeutics to improve clinical outcomes and overcome the rise in antibiotic resistance.

While NMEC strains share many similarities, including a propensity to express the K1 capsule antigen, there is a high degree of variability in virulence
factor expression (20, 38). To that end we investigated differences in immune stimulation and bacterial pathogenicity of two strains of NMEC. Both NMEC O18 and NMEC RS218 were originally cultured from cerebrospinal fluid of infants with meningitis (191, 192). Both strains have been fully sequenced, and both are O18:H7:K1 *E. coli* (191, 192). One important difference is that NMEC RS218 is known to express the toxins CNF-1 and HlyA, while NMEC O18 does not. CNF-1 is a toxin which 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 (193, 194). CNF-1 has also been shown to aid NMEC in crossing the blood-brain barrier (86).

HlyA is a pore-forming toxin expressed by approximately 30% of NMEC isolates which has been associated with increased severity of uropathogenic *E. coli* (UPEC) infection. HlyA has been shown to interact with several immune pathways, including the NLRP3 inflammasome and purinergic receptor pathways, in the context of UPEC infection (113, 114, 149, 150, 187). While these interactions could contribute to increased pathogenicity of HlyA+ bacteria, under certain conditions they could 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 (97). Therefore, it is not surprising that HlyA has been associated with increased NLRP3 activation (113, 114). Following activation, NLRP3, like other inflammasomes, activates caspase-1, which in turn cleaves pro-
IL-1β to its active forms (91, 92). Activated caspase-1 also cleaves 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 (94). Accumulation of GSDMD pores in the cell membrane can also lead to the lytic form of cell death known as pyroptosis (95).

P2XRs are membrane-bound, cation-selective, ligand-gated ion channels that are activated primarily by extracellular ATP (126). Large quantities of ATP are released into the extracellular space following cell damage, such as from HlyA pores (120-122). 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 (121). We therefore assess how differential activation of P2XRs impact the outcome of infection in neonatal mice.

RESULTS

NMEC O18 infection results in markedly higher bacterial loads compared to NMEC RS218 infection of neonatal mice.

It has previously been shown that NMEC O18 is significantly more virulent than NMEC RS218 in a neonatal rat model of infection (195). To determine if NMEC O18 was also more virulent in a neonatal mouse model, we infected 3-day-old C57BL/6 mice with \( \sim 10^4 \) NMEC O18 or NMEC RS218 i.p. The mice were euthanized 18 hours post-infection and blood, peritoneal lavage fluid and brains were collected to determine bacterial burden. We found that mice infected with
NMEC O18 had ~100,000-fold higher bacterial loads in all three tissues than mice infected with NMEC RS218. (Fig. 16A-C).

**NMEC RS218 induces significantly more IL-1β secretion and macrophage cell death than NMEC O18.**

HlyA and CNF-1 are associated with increased disease severity in response to UPEC infection (196-199), and expression of CNF-1 by NMEC strains aids in crossing the BBB (86). It is therefore interesting that infection with NMEC RS218, which carries both of these toxins, results in lower bacterial loads than infection with the non-toxin-producing NMEC O18 strain. We hypothesized that the toxins may be triggering protective innate immune pathways in the context of NMEC RS218 infection which contribute to the improved bacterial clearance of this strain compared to NMEC O18.

CNF-1 has been shown to facilitate crossing of the BBB; however, it is also known to increase nonspecific endocytosis of material by macrophages (193). To determine the potential role of CNF-1 in NMEC RS218 infection we assessed bacterial uptake and killing by peritoneal macrophages. We infected macrophages with either NMEC RS218 or NMEC O18 for 1 hour and then treated with gentamicin to kill extracellular bacteria. The cells were subsequently lysed either 1.5- or 5-hours post-infection to determine the number of bacteria that were phagocytosed, and also whether the bacterial strains were killed differentially by the macrophages once in the cells. We found that, as expected, there were higher levels of NMEC RS218 within the cells than NMEC O18 (Fig. 17A). However, once in the cells, there was no apparent difference in the survival of either bacteria (Fig. 17B).
Figure 16. NMEC O18 infection results in markedly higher bacterial loads compared to NMEC RS218 infection of neonatal mice.

3-day-old wildtype mice were i.p. infected with ~10^4 NMEC RS218 or NMEC O18. Mice were euthanized 18 hours post-infection and brain homogenate (A), blood (B), and peritoneal lavage (C) were plated on MacConkey agar to determine bacterial loads (n = 9-10 per group). Three NMEC O18 infected mice were dead at the time of necropsy and blood could not be collected. Data are combined from 2 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at P < 0.05.
We assessed secretion of IL-6 by peritoneal macrophages infected with either NMEC O18 or NMEC RS218. Interestingly, we found that macrophages infected with either bacteria secreted similar levels of IL-6 following infection (Fig. 18A). We next measured secretion of IL-1β and found that NMEC O18-infected macrophages secreted significantly less IL-1β compared to NMEC RS218-infected cells (Fig. 18B). IL-1β requires a two-step process for activation and secretion. In the first step, IL-1β mRNA is induced following TLR activation, and pro-IL-1β is produced. The second step requires activation of inflammasomes, which activate caspase-1 to cleave pro-IL-1β to its active form. Once cleaved, IL-1β can then be secreted from the cell (102). We previously found that IL-1β secretion in response to NMEC RS218 is both TLR4- and NLRP3- dependent (166). To determine whether the differential secretion of IL-1β by NMEC O18 and NMEC RS218 was due to differences in mRNA induction or inflammasome activation, we measured mRNA induction compared to uninfected macrophages, and found that both NMEC O18 and NMEC RS218 induced a similar amount of IL-1β mRNA (Fig. 18C), indicating that the difference in IL-1β secretion is more likely due to inflammasome activation. This was particularly interesting since HlyA is known to activate the NLRP3 inflammasome. NLRP3 activation could therefore serve as a differential anti-virulence immune response, and 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 protection of wildtype neonatal mice against NMEC RS218 infection (166), suggesting a need to continue searching for other differential responses between the two bacterial strains.
Figure 17. Peritoneal macrophages phagocytose significantly more NMEC RS218 than NMEC O18.

Wildtype thioglycolate-elicited peritoneal macrophages were infected with either NMEC RS218 or NMEC O18 at an MOI of 10 or 100. Cells were washed and treated with gentamicin to remove extracellular bacteria 1-hour post-infection. Cells were then lysed either 1.5-hours (A) or 5-hours (B) post-infection and lysates were plated on MacConkey agar to determine intracellular bacterial loads. In (B) % E. coli survival was calculated compared to bacterial loads at the 1.5-hour time point. Data are representative of 3 independent experiments with 3 wells per group. *, P < 0.05. NS = not significant. The error bars represent SD of the mean.
Figure 18. NMEC RS218, but not NMEC O18 induces significant macrophage cell death and IL-1β secretion.
Wildtype thioglycolate-elicited peritoneal macrophages were infected with either NMEC RS218 or NMEC O18 at an MOI 0.1. Supernatants were harvested 5-hours post-infection and analyzed for IL-6 (A) and IL-1β (B) secretion by ELISA. In (C), cells were lysed for RNA purification and subsequent qRT-PCR. IL-1β expression was calculated as fold change compared to mock infected cells $2^{\Delta\Delta Ct}$. In (D), cell death was measured by LDH assay. Data are representative of 2-3 independent experiments with 3 wells per group. Means with the same letter are not statistically different from each other. *, $P < 0.05$. N.S. = not significant. The error bars represent S.D. of the mean.
In addition to IL-1β secretion, inflammasome activation also leads to the cleavage of GSDMD. Following cleavage, the N-terminal fragment of GSDMD is able to polymerize within the cell membrane to create pores through which cytokines, particularly IL-1β and IL-18 are able to leave the cell (96, 200). Furthermore, these pores can ultimately lead to destabilization of the cell membrane, resulting in the lytic form of cell death known as pyroptosis (95). 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 (Fig. 18D).

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 (153, 154), prior to infection with NMEC RS218. We found that cytochalasin-D led to significantly reduced intracellular bacteria (Fig. 19A). Secretion of IL-1β and IL-6 were also significantly decreased, but not completely abrogated in cytochalasin-D treated macrophages (Fig. 19B and C), 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 (Fig. 19D), 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 and cell death during NMEC RS218 infection.
Figure 19. Blocking uptake of NMEC RS218 by macrophages significantly decreases IL-1β and IL-6 secretion, but has no effect on cell death.

Wildtype thioglycolate-elicited peritoneal macrophages were treated with cytochalasin-D prior to infection with NMEC RS218. Supernatants were harvested 5 hours post-infection. Cells were washed, lysed and plated on MacConkey agar to quantify intracellular bacteria (A). Supernatants were analyzed for IL-6 (B) and IL-1β (C) secretion by ELISA. In (D), cell death was measured by LDH assay. Data are representative of 2 independent experiments with 3 wells per group. *, P < 0.05. N.S. = not significant. The error bars represent SD of the mean.
To further characterize the cell death in response to NMEC RS218 infection, we next pre-treated peritoneal macrophages with either glycine, which is known to stabilize cell membranes and limit lytic cell lysis (156, 157), necrosulfonamide, which inhibits GSDMD from polymerizing in the cell membrane (158), or KCl, which is known to block NLRP3 activation by limiting potassium efflux (97, 201). Combined treatment of LPS with nigericin, a potassium ionophore known to activate the NLRP3 inflammasome pathway (201), 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 either LPS/nigericin treatment or NMEC RS218 infection, but significantly reduced cell death (Fig. 20A and B). Interestingly, while treatment with either necrosulfonamide or KCl abrogated secretion of IL-1β following both LPS/nigericin treatment and NMEC RS218 infection, and significantly inhibited cell death following LPS/nigericin treatment, neither NLRP3 nor GSDMD inhibition had a significant effect on cell death following NMEC RS218 infection (Fig. 20A and B). To further confirm that cell death was independent of NLRP3 inflammasome activation, peritoneal macrophages from wildtype, NLRP3−/− and Caspase 1/11−/− mice were infected with NMEC RS218. We found no difference in cell death between the three groups (Fig. 20C), further indicating that macrophage cell death in response to NMEC RS218 infection is not inflammasome-mediated.
Figure 20. Macrophage cell death in response to NMEC RS218 infection is not dependent on the NLRP3 inflammasome or gasdermin D.

Wildtype thioglycolate-elicited macrophages were treated with DMSO (vehicle control), glycine, necrosulfonamide, or KCl 1-hour prior to infection with NMEC RS218 at an MOI of 0.1 or 1 or treatment with LPS/nigericin. Supernatants were collected 5-hours post infection and analyzed for IL-1β secretion (A) by ELISA and relative % cell death (B) by LDH assay. In (C), WT, NLRP3<sup>-/-</sup>, and Caspase 1/11<sup>-/-</sup> peritoneal macrophages were infected with NMEC RS218 and relative cell death was determined using an LDH assay. Data are representative of 2-3 independent experiments with 3 wells per group. Means with the same letter are not statistically different from each other. N.S. = not significant. The error bars represent SD of the mean.
Macrophage cell death in response to NMEC RS218 infection requires purinergic receptor signaling, but is independent of NLRP3 inflammasome activation.

The HlyA pore has been associated with release of ATP into the extracellular space and subsequent activation of purinergic receptors which can also lead to cell death via caspase-dependent and -independent mechanisms (125, 129-131). To assess the role of purinergic receptors in vitro we pre-treated peritoneal macrophages with brilliant blue G (BBG), which is most specific for P2X7, but can inhibit other P2X receptors at higher concentrations (202). Interestingly, we found that BBG-treatment significantly decreased cell death, but did not have a significant impact on IL-1β secretion (Fig. 21A and B), further uncoupling the NLRP3-dependent IL-1β response from the cell death response.

It has previously been suggested that HlyA interacts with the pannexin-1 receptor to release ATP from the cell (147). 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 (203). We found that in contrast to BBG, BB-FCF did not significantly impact IL-1β secretion or cell death following NMEC RS218 infection (Fig. 21C and D), suggesting that NMEC RS218 is able to induce release of ATP and purinergic receptor activation in the absence of pannexin-1 signaling.

P2XR-dependent cell death is often attributed to activation the NLRP3 inflammasome and subsequent pyroptosis; however, we already showed that the cell death in response to NMEC RS218 is independent of NLRP3 activation (Fig. 20). Others have demonstrated that P2XR activation can also induce apoptosis, and it has further been suggested that the necroptotic pathway could be activated
Figure 21. Macrophage cell death, but not IL-1β secretion in response to NMEC RS218 is dependent on P2XRs.
Wildtype thioglycolate-elicited macrophages were treated with BBG, KCl, or BB-FCF (FCF) 1-hour prior to infection with NMEC RS218 at an MOI of 0.1 or 1 or treatment with LPS/nigericin. Supernatants were collected 5 hours post infection and analyzed for IL-1β secretion (A and C) by ELISA and relative % cell death (B and D) by LDH assay. Data are representative of 2-3 independent experiments with 3 wells per group. Means with the same letter are not statistically different from each other. NS = not significant. The error bars represent SD of the mean.
downstream of P2XR. We therefore assessed the role of both apoptosis and necroptosis in NMEC RS218 induced cell death. To assess the contribution of apoptosis to cell death, we infected macrophages lacking caspase-8 or caspase-1, -11 and -8, and found that cell death was not caspase-8 dependent (Fig. 22A). 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 (Fig. 22B). Finally, it is known that pyroptosis, necroptosis and apoptosis are highly interconnected and frequently compensatory (204). We therefore assessed whether blocking all three pathways simultaneously would have a significant impact on cell death. By using the Caspase 8/1/11−/− macrophages 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 (Fig 22C), suggesting that the cell death induced by NMEC RS218 is more likely due to non-programmed necrosis.

**Induction of death is associated with HlyA+ NMEC strains**

One of the major differences between NMEC RS218 and NMEC O18 is the production of HlyA by NMEC RS218, but not NMEC O18. HlyA is associated with induction of macrophage death during UPEC infection (175, 187). To determine if NMEC strain-dependent cell death was also specifically associated with HlyA+ strains we infected 6 additional HlyA− NMEC strains and 7 additional HlyA+ strains.
Figure 22. P2XR-dependent cell death in response to NMEC RS218 infection is not caspase or RIPK1 dependent.

In (A), thioglycolate-elicited macrophages from WT, Caspase 8\(^{fl/fl}\)LysM\(^{cre}\) (Casp8\(^{-/-}\)) and Caspase 8\(^{fl/fl}\)LysM\(^{cre}\)Caspase 1/11/8\(^{-/-}\) (Casp1/11/8\(^{-/-}\)) mice were infected with NMEC RS218. In (B), WT macrophages were treated with necrostatin-1 one hour prior to infection. In (C), Casp1/11/8\(^{-/-}\) macrophages were treated with necrostatin-1, z-VAD, or both one hour prior to infection. Supernatants were collected 5 hours post-infection to measure relative % cell death by LDH assay. Data are representative of 2-3 independent experiments with 3 wells per group. Means with the same letter are not statistical different. NS = not significant. The error bars represent SD of the mean.
These strains were chosen to represent various phylotypes and O-antigen groups (Table 1). As expected, the 6 hemolytic HlyA+ strains induced cell death in greater than 39% of infected macrophages, and this cell death was P2XR-dependent (Fig. 23). In contrast, HlyA- strains induced cell death in less than 7% of macrophages (Fig. 23), suggesting that HlyA is responsible for P2XR-dependent cell death. Interestingly, the one HlyA+, non-hemolytic strain, NMEC 42, induced roughly half as much cell death as the hemolytic HlyA+ strains.

*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 x 10^3 CFU NMEC RS218. We found that BBG treatment significantly decreased survival times, and increased bacterial loads in the blood of NMEC RS218-infected mice (Fig. 24A and B). We next infected BBG or PBS pre-treated neonates with NMEC O18, and found no difference in survival or bacteremia between the two groups (Fig. 24C and D). To ensure that any potential protective effects of P2XRs were not masked by the increased virulence of NMEC O18 compared to NMEC RS218, 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 (20 hours for 5000 CFU vs. 24 hours for 50 CFU), there was still no significant difference in survival time between control pups and those treated with BBG (Fig. 24E and F). These results suggest that P2XR activation plays a protective role in NMEC RS218 infection, but not NMEC O18 infection.
Figure 23. HlyA+ NMEC strains induce significantly more P2XR-dependent cell death than HlyA− NMEC strains.

Wildtype thioglycolate-elicited macrophages were infected with NMEC strains at an MOI of 0.1. Some wells were treated with BBG prior to infection. Data from macrophages infected with HlyA+ strains are on the left side of the figure and data from macrophages infected with HlyA− strains are on the right side of the figure. * P < 0.05 compared to untreated macrophages infected with the same NMEC strain. The error bars represent SD of the mean.
Figure 24. P2XR Signaling is protective during NMEC RS218 infection, but not NMEC O18 infection in neonatal mice.

3-day-old mice were treated with either PBS or BBG ~2 hours 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 500 CFU NMEC O18 (E and F) (low-dose, n = 10/group). Blood was taken 18 hours 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 hours post-infection, and blood could not be collected. Data are combined from 2 or 3 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at $P < 0.05$. 

A. % Survival vs. hours post-infection (PBS vs. BBG, $P < 0.001$)

B. Log$_{10}$ CFU/mL Blood (PBS vs. BBG, $P < 0.01$)

C. % Survival vs. hours post-infection (PBS vs. BBG, $P = 0.754$)

D. Log$_{10}$ CFU/mL Blood (PBS vs. BBG, $P = 0.534$)

E. % Survival vs. hours post-infection (PBS vs. BBG, $P = 0.399$)

F. Log$_{10}$ CFU/mL Blood (PBS vs. BBG, $P = 0.186$)
P2X7R is one of the most widely studied P2XRs, and has been found to be important in numerous infection models (136, 137, 149, 150). Furthermore, BBG is often used at low doses as a P2X7R specific inhibitor. To determine if the effects of BBG in our model were due to specific inhibition of P2X7R, we first collected P2X7−/− peritoneal macrophages, and infected them with NMEC RS218. Interestingly, we found that there was a modest but significant difference in cell death between wildtype and P2X7−/− cells, but only at the highest MOI (Fig. 25A). We also treated P2X7−/− macrophages with BBG and found a marked decrease in cell death among the BBG-treated cells (Fig. 25B), indicating that effect of BBG-treatment in our system is not solely due to inhibition of P2X7R. 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 hours post-infection, and tissues were collected to determine bacterial loads. We found that the bacterial burden was significantly higher 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 (Fig. 26A-D). This suggests that the effect of BBG on outcomes of NMEC RS218 infection in neonatal mice is not solely dependent on specific inhibition of P2X7R, and that other P2XRs either alone or in concert are likely responsible for the protective effects of P2XRs in our model. We further determined that NLRP3 is not essential for P2XR-dependent protection, as NLRP3−/− pups treated with BBG had significantly increased bacterial loads in the brain, peritoneum and spleen compared to PBS-treated pups (Fig. 27A, C and D). This is in agreement with our earlier finding that NLRP3 activity is not required.
Figure 25. P2X<sub>7</sub>R is not solely responsible for macrophage death <em>in vitro</em> following infection with NMEC RS218.

Peritoneal macrophages harvested from wildtype (WT) or P2X<sub>7</sub>R<sup>-/-</sup> mice were infected with NMEC RS218. Supernatant were collected 5 hours post-infection to assess cell death using an LDH assay. In (B), P2X<sub>7</sub>R<sup>-/-</sup> cells were pre-treated with PBS or BBG ~1-hour prior to infection. Data are representative of 2 independent experiments with 3 wells/group. *, P < 0.05. The error bars represent SD of the mean.
Figure 26. P2X₇R is not solely responsible for the protective effect of P2XR activation in vivo following infection with NMEC RS218.

3-day-old P2X₇⁻/⁻ mice were pre-treated with PBS or BBG ~2 hours prior to infection with NMEC RS218. Mice were euthanized 18 hours post-infection and brain homogenate (A), blood (B), peritoneal lavage (C), and spleen homogenate (D) were plated on MacConkey agar to determine bacterial loads. n = 12-13 per group. Data are combined from 3 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at $P < 0.05$. 
Figure 27. NLRP3 activity is not required for P2XR-dependent protection.
3-day-old NLRP3−/− mice were pre-treated with PBS or BBG ~2 hours prior to infection with NMEC RS218. Mice were euthanized 18 hours post-infection and brain homogenate (A), blood (B), peritoneal lavage (C), and spleen homogenate (D) were plated on MacConkey agar to determine bacterial loads. n = 11 per group. Two BBG-treated mice were found dead at the time of necropsy, and blood could not be collected. Data are combined from 4 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at $P < 0.05$. 

\[
P = 0.018 \quad P = 0.111 \quad P < 0.001 \quad P < 0.001
\]
for P2XR-dependent cell death from NMEC RS218-infected macrophages (Fig. 21). It is important to note that bacterial loads in the blood were not significantly different between groups (Fig. 27B), which could indicate a partial dependence on NLRP3 \textit{in vivo}.

\textbf{The protective effect of P2XR signaling requires the presence of monocytes and macrophages, but not neutrophils.}

We found that bacterial loads in the brain and peritoneum were similar between PBS- and BBG-treated groups 6 hours following NMEC RS218 infection (Fig. 28A and B), and only 10-fold higher in the BBG treated animals at 12 hours post-infection (Fig. 28C and D). We, therefore, used these time points to evaluate cytokine and chemokine production in order to evaluate the early immune response leading to the significantly different bacterial loads noted at 18 hours post-infection. At 6 hours post-infection, when bacterial counts were similar, Control mice had significantly higher IFN-\(\gamma\), IL-1\(\beta\), IL-6, IL-17, MIG and MCP-1 (Fig. 29A-F), indicating a diminished early pro-inflammatory response in BBG treated animals. By 12 hours post-infection, BBG-treated mice had similar levels of IFN-\(\gamma\), IL-1\(\beta\), IL-6, IL-17 and MIG, and significantly elevated MCP-1, KC, MIP-1\(\alpha\) and TNF-\(\alpha\) (Fig. 29). However, this is not completely unexpected since by this point bacterial loads were significantly higher in BBG-treated animals and the increased bacterial burden likely contributes to increased cytokine production. No differences were noted in IL-10 or IL-4 production at either time point (Data not shown).
Figure 28. Bacterial loads are similar between PBS- and BBG- treated mice 6 hours post-infection with NMEC RS218.
3-day-old wildtype mice were pre-treated with PBS or BBG ~2 hours prior to infection with NMEC RS218. Mice were euthanized 6 hours (A and B) or 12 hours (C and D) post-infection and brain homogenate (A and C), and peritoneal lavage (B and D) were plated on MacConkey agar to determine bacterial loads. n = 10-11 per group. Data are combined from 3 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at $P < 0.05$. 
Figure 29. Treatment with BBG alters inflammatory cytokine profiles in peritoneal lavage following NMEC RS218 infection.

3-day-old mice were pre-treated with PBS or BBG ~2 hours prior to infection with NMEC RS218. Mice were euthanized 6 hours or 12 hours 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 line represents the sample median. Significance is set at $P < 0.05$. 

- IFN-γ
- IL-1β
- IL-6
- IL-17
- MIG
- MCP-1
- KC
- MIP-1α
- TNF-α
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 (87, 88). Furthermore, P2XRs play a key role in neutrophil activation and function, specifically playing a role in chemotaxis, extravasation, cytokine release and prolonging cell survival (122, 139, 140). IL-1β, IL-6, and IL-17, all of which were decreased in the lavage samples from BBG-treated mice at 6 hours post-infection (Fig. 29B-D), are all associated with recruitment of neutrophils to the site of infection (99, 205-207). To determine if P2XR-dependent protection requires neutrophils, we depleted neutrophils from neonatal mice prior to PBS or BBG treatment and subsequent infection with NMEC RS218. Depletions were confirmed by flow cytometry. While neutrophils made up approximately 32.9% of cells in the peritoneum of infected, isotype-treated animals, neutrophils only accounted for approximately 0.15% of peritoneal cells in anti-Ly6G-treated mice (data not shown). Interestingly, following neutrophil depletion, BBG-treated mice still had significantly higher bacterial loads in brain homogenate, blood, peritoneal lavage and spleen homogenate at 18 hours post-infection (Fig. 30A-D), suggesting that the protective effect of P2XR activation during NMEC RS218 infection is not neutrophil-dependent.

Macrophages and monocytes are another important component of the innate immune response, and are also known to be recruited and activated in response to P2XR signaling (121). MCP-1, which we found to be diminished in BBG-treated mice at 6 hours post-infection (Fig 29F), is an important chemoattractant for monocyte migration to the site of infection (208). We have also
Figure 30. The protective effect of P2XR signaling during NMEC RS218 infection does not require neutrophils.

Wildtype neonatal mice were depleted of neutrophils with anti-Ly6G (clone 1a8) on post-natal days 1-3 and then pre-treated with PBS or BBG ~2 hours prior to infection with NMEC RS218 on post-natal day 3. Mice were euthanized 18 hours post-infection and brain homogenate (A), blood (B), peritoneal lavage (C) and spleen homogenate (D) were plated on MacConkey agar to determine bacterial loads. n = 21 per group. Data are combined from 4 independent infections. The solid line represents the sample median. The dashed line represents the limit of detection. Significance is set at $P < 0.05$. 

$P = 0.069$

$P = 0.003$

$P = 0.002$

$P = 0.001$
shown that P2XRs induce macrophage cell death during *in vitro* infection with NMEC RS218 (Fig. 21), suggesting that P2XRs on macrophages are activated by NMEC RS218 infection. To determine if P2XR-dependent protection required monocytes/macrophages, we treated pups with clodronate liposomes one day prior to PBS/BBG treatment and NMEC RS218 infection. While inflammatory monocytes and macrophages accounted for approximately 10.2% and 22.8% of cells in the peritoneum of infected mice that did not receive clodronate respectively, they only accounted for 3.5% and 3.1% of peritoneal cells in clodronate-treated animals (data not shown). We did not see a significant difference in bacterial loads in the lavage samples or spleens of PBS- and BBG- treatment groups (Fig. 31C and D), indicating that monocytes/macrophages are required for P2XR-dependent protection. Interestingly, we found that blood and brain homogenate from BBG-treated macrophage-depleted mice actually had significantly lower bacterial loads than PBS-treated controls (Fig. 31A and B). This could indicate a deleterious role of P2XR signaling in the absence of macrophages/monocytes.

To determine whether the protective effect of P2XR signaling was due to stimulation of increased phagocytosis or bacterial killing by macrophages we next treated peritoneal macrophages with PBS or BBG prior to infection with NMEC RS218. The ability of the macrophages to phagocytose bacteria was assessed at 1.5 hours post infection and bacterial killing was assessed at 5- and 24-hours post-infection via a gentamicin-killing assay. While there was a significant decrease in phagocytosis by BBG-treated cells at the highest MOI (Fig. 32A), there was no
Figure 31. The protective effect of P2XR signaling during NMEC RS218 infection requires macrophages and monocytes.

2-day-old wildtype mice were depleted of macrophages/monocytes with clodronate liposomes and then pre-treated with PBS or BBG ~2 hours prior to infection with NMEC RS218 on post-natal day 3. Mice were euthanized 16-18 hours 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 = 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 line represents the sample median. The dashed line represents the limit of detection. Significance is set at $P < 0.05$. 
difference in the ability of cells to kill the bacteria at any time point (Fig. 32B and C), suggesting that P2XRs are not inducing protection by improving the phagocytic or bacteria-killing capacity of macrophages. While more research is needed to determine the precise mechanism by which P2XR signaling induces a protective response, the data presented here indicate this protection requires the presence of monocytes/macrophages, but not neutrophils.

DISCUSSION

Despite significant variability in virulence traits present on 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 (27, 76, 86). However, in order to cause meningitis, NMEC must first evade the host immune response in order to reach a threshold bacteremia (25, 27, 75). The most well studied NMEC virulence factor in this context is OmpA. Expression of OmpA is associated with subverting the complement cascade, preventing maturation of dendritic cells, and suppressing the bacterial killing ability of macrophages and neutrophils (66, 83, 85, 209). However, only 66% of NMEC isolates express OmpA, and this is not significantly different from the distribution human fecal E. coli, suggesting that the presence of OmpA is not a main determinant factor for NMEC virulence (20). 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 other
Figure 32. P2XR signaling does not significantly contribute to phagocytosis or bacterial killing by peritoneal macrophages during NMEC RS218 infection.
Wildtype thioglycolate-elicited peritoneal macrophages were treated with BBG prior to infection with NMEC RS218 at an MOI of 1, 10 or 100. Cells were washed and treated with gentamicin to remove extracellular bacteria 1 hour post-infection. Cells were then lysed either 1.5 hours (A), 5 hours (B), or 24 hours (C) post-infection and lysates were plated on MacConkey agar to determine intracellular bacterial loads. In (B) and (C) % E. coli survival was calculated compared to bacterial loads at the 1.5-hour time point. Data are representative of 2 independent experiments with 3 wells per group. *, P < 0.05. The error bars represent SD of the mean.
ExPEC infection, but not NMEC. Better understanding of these factors could lead to improved diagnostic and prognostic abilities. 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, a HlyA- NMEC strain, did not (Figs. 18D and 21B). P2XR activation has previously been associated with HlyA expression on UPEC (147). We therefore infected peritoneal 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 the 7 additional HlyA+ NMEC strains, while HlyA- strains induced little to no cell death (Fig. 23). NMEC 42 expresses 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 (187). Deletion of the cof gene resulted in significantly decreased HlyA secretion, as well as significantly reduced cell lysis and IL-1β secretion (187). Similarly, we found that NMEC 42 induced only half as much P2XR-dependent cell death as other HlyA+ NMEC strains, 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 release of IL-1β and cell death via pyroptosis (129). However, we found that NMEC RS218 induced NLRP3-
dependent IL-1β secretion does not require P2XR activation (Fig. 21), and P2XR-dependent cell death does not require NLRP3 activation (Fig. 20), suggesting an uncoupling of P2XR and NLRP3 responses 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 (114). It is therefore possible that NMEC RS218 expresses high levels of HlyA and this is the reason for the uncoupled NLRP3 and P2XR responses. Further studies would be needed to correlate the requirement of NLRP3 for cell death with HlyA expression levels in NMEC strains. Interestingly, the same study which demonstrated this correlation in UPEC, also found that infection with the UPEC strain with low HlyA expression resulted in significantly decreased bacterial loads in the bladder of mice when compared to a mutant strain lacking HlyA, however this protective effect of HlyA was lost when HlyA was overexpressed in the same bacterial strain (114).

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 hours post-infection and significantly decreased survival (Fig. 24A and B). In contrast, BBG had no impact on the outcome of NMEC O18 infection (Fig. 24C-F). This finding, in combination with the observation that NMEC O18 infection results in significantly higher bacterial burdens than NMEC RS218 infection (Fig. 16), suggests that NMEC strain-dependent activation of P2XRs is protective during NMEC infection, and factors that activate this pathway, such as
HlyA, may potentially function as anti-virulence factors leading to improved bacterial clearance in the context of NMEC infection.

The effect of P2XR signaling has often been attributed to its role in the activation of NLRP3 and subsequent production of IL-1β (129). However, neonates have a diminished NLRP3 response compared to adults (109). We also showed that IL-1 signaling is only protective in neonatal mice in the absence of NO (166), and that P2XR activation and NLRP3 activation appear to be uncoupled during in vitro NMEC RS218 infection (Fig. 21). 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 (Fig. 26).

P2XR activation can have a number of effects on various innate immune cells in vivo, including both neutrophils and macrophages (121). We found that BBG-treated neonates had significantly increased IL-1β, IL-6, and IL-17 in peritoneal lavage fluid following NMEC RS218 infection (Fig. 29B-D), all of which are associated with neutrophil chemotaxis (99, 205, 206). We treated neutrophil-depleted pups with PBS or BBG prior to infection with NMEC RS218 to determine whether neutrophils were required for P2XR-dependent protection. We found that BBG-treated pups still had significantly higher bacterial loads, even following neutrophil depletion, indicating that the protective effect of P2XR does not require neutrophil function (Fig. 30). Interestingly, in a small control study, neutrophil-depleted mice had similar bacterial loads in all tissues as mice treated with an
isotype control antibody prior to infection (data not shown), suggesting that neutrophils may not provide significant protection in our model. The role of neutrophils in NMEC infection has previously been assessed with conflicting outcomes. One study found neutrophils to be extremely important in bacterial clearance and prevention of mortality during neonatal infection in an i.p. infection model using an unspecified K1 *E. coli* strain (88). Another study found a detrimental effect of neutrophils on the outcome of a neonatal i.n. infection model using a derivative of the NMEC RS218 strain (89). As we also observed minimal effect of neutrophil depletion during our NMEC RS218 infection, this may indicate a bacterial strain-dependent effect of neutrophil activation and protection during NMEC infection rather than a route/tissue-dependent effect.

Our peritoneal cytokine analysis also showed significantly decreased MCP-1 in BBG-treated neonates early in infection (Fig. 29F). This finding, along with our finding that P2XRs are activated on macrophages *in vitro*, suggested that monocytes and macrophages could also potentially be involved in P2XR dependent protection of NMEC RS218-infected neonates. Interestingly, we found that following monocyte/macrophage depletion, BBG treatment did not significantly alter bacterial loads in the peritoneum or spleens of NMEC RS218-infected pups (Fig. 31), indicating that P2XR signaling is only protective in the presence of macrophages and monocytes. Bacterial loads in all monocyte/macrophage-depleted mice were also higher than what we would expect of non-depleted animals, further suggesting a protective role of monocytes/macrophages in NMEC RS218 infection. This is in contrast to the one previous study of macrophages in
NMEC infection, where macrophage depletion using α-carrageenan led to significant decrease in bacterial loads following i.n. infection with a rifampicin-resistant derivative of NMEC RS218 (84). This effect was attributed to OmpA-dependent evasion of macrophage bactericidal properties, allowing the macrophages to become a niche for bacterial replication (84, 85). 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 (210, 211). Alternatively, treatment with clodronate liposomes induces macrophage apoptosis, and is not believed to activate macrophages prior to depletion (211). The inability of liposomes to efficiently cross the vascular endothelium does potentially limit the depletion efficiency and makes choosing the route of administration extremely important (211). In our study, we administered clodronate liposomes i.p., the location of our primary infection, and we did see a significant reduction in macrophages/monocytes in the peritoneum. It is, however, possible that macrophage/monocyte were not depleted, or were less efficiently eliminated in other tissues. Interestingly, while we found no difference in bacterial loads in the peritoneum or spleen (Fig. 31C and D), BBG-treated mice had significantly decreased bacterial loads in the brain and blood (Fig. 31A and B). This could
represent a general deleterious effect of P2XR signaling in the absence of monocytes/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 peritoneal macrophages (Fig. 32), 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 (121). We will therefore address the effects of P2XRs on macrophage migration and cytokine production in future studies.

Taken together, these data indicate that bacterial strain-dependent P2XR activation is protective during NMEC infection, and that macrophages/monocytes, but not neutrophils are required for this protection. Our data further suggests that this strain-dependent effect is due to expression of HlyA. More research is required to determine how P2XRs mediate macrophage/monocyte-dependent protection (i.e., via increased recruitment or activation), as well as what specific P2XRs are involved, and whether 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 take 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 again serve as prognostic indicators, and potentially aid in the development of improved treatment plans for these infants.
CHAPTER V

DISCUSSION

Bacterial meningitis and sepsis remain important causes of morbidity and mortality in newborn infants with premature infants being the most at risk (21-23, 40). *E. coli* has historically been the second leading cause of bacterial meningitis behind GBS; however, as the use of peripartum antibiotics to prevent GBS becomes more common, some institutions are seeing a relative rise in the rate of *E. coli*-associated disease (34, 35). While improved antibiotic treatments have significantly decreased mortality rates among infants with meningitis, these children are still at an increased risk of developing life-long neurologic impairments compared to healthy infants (22, 40-42). Furthermore, rising antibiotic resistance among NMEC isolates has complicated the treatment of these infections (43-49). Immune-based therapeutics could be a promising addition to current treatment plans if they could be engineered to augment the ability of the host immune response to clear the infection while limiting deleterious responses and tissue damage; however, these types of therapeutics require an in-depth understanding of both protective and maladaptive responses to individual pathogens. This has proven challenging in neonatal infections due to age-dependent alterations in immune function during the neonatal period many of which are still not completely understood (50, 56, 60-63, 67, 68). Studying the immune response to NMEC is made even more challenging by the variability in virulence factor profiles between...
NMEC isolates (1, 2, 10). Here we explore the effect of both host age and bacterial virulence factors on the function of macrophages in NMEC infection. Specifically, we show that age-dependent IL-1 secretion and HlyA-dependent P2XR signaling affect the outcome of NMEC infection in a neonatal mouse model.

**Age as a factor in NMEC infection**

The neonatal immune system is altered compared to the immune system of adults, with important differences in both the innate and adaptive immune responses (50, 53, 55-58). While it is clear that these differences contribute to the increased susceptibility of neonates to infection, the mechanisms accounting for enhanced susceptibility remain unclear. Here, we demonstrated that endogenous IL-1 signaling is protective in an adult mouse model of NMEC-induced meningitis (Fig. 10), but we did not see an effect of IL-1R blockade on the outcome of NMEC-induced neonatal sepsis and meningitis (Fig. 11). IL-1β secretion is known to be impaired in neonates, and could serve as a factor contributing to this differential response (109-111). It has also been demonstrated that administration of exogenous IL-1 can be protective during neonatal NMEC infection (184) further suggesting that age-dependent differences in IL-1 processing may be responsible for the lack of response to IL-1R blockade. We and others have previously shown that NO can block IL-1β cleavage and secretion via S-nitrosylation of NLRP3 (152, 172). Furthermore, human infants are known to have higher baseline NO levels in the blood (78), and neonatal mouse microglial cells display increased iNOS expression in response to LPS stimulation compared to microglial cells from adult mice *in vitro* (80). We also found that endogenous IL-1 signaling is protective
during neonatal infection in the absence of iNOS (Fig. 15), and that NOS2−/−
neonates secrete higher levels of bioactive IL-1 into the peritoneum, despite similar
bacterial loads at 12 hours post-infection (Fig. 14). Therefore, we propose that
increased NO production by neonates inhibits the secretion of bioactive IL-1 by
blocking NLRP3 activation, ultimately limiting the potentially protective role of IL-1
signaling during neonatal NMEC infection. This same mechanism may also serve
as a general mechanism for increased susceptibility of infants, particularly
premature infants, to infection.

Although we did not assess age as a factor in the role of P2XR in response
to NMEC infection, there is evidence that purinergic receptor activation may be
somewhat altered in neonates. While it has previously been shown that blood
macrophages from both neonates and adult humans express similar levels of P2X7
(109), neonatal blood also contains higher concentrations of enzymes that break
down ATP to adenosine (145). This could result in a response skewed towards
activation of the anti-inflammatory P1Rs rather than the pro-inflammatory P2Rs.
We showed here that inhibition of P2XR signaling is detrimental during neonatal
NMEC RS218 infection (Fig. 24A) suggesting that during infection, ATP reaches
concentrations sufficient to activate P2XRs during infection in neonatal mice. We
also demonstrated an uncoupling of P2XR and NLRP3 activation both in vitro and
in vivo (Figs. 20 and 27). This is important since the protective effects of P2XR in
certain infections have been attributed to the secondary activation of NLRP3 (129).
In these infections, P2XR-dependent protection may be attenuated in neonates as
a result of diminished NLRP3 activation. Overall, more research is needed to fully understand the contribution of P2XR signaling to neonatal infection.

**Alpha-hemolysin as an anti-virulence factor in NMEC infection**

HlyA is a pore-forming toxin whose role in UPEC infection has been well-characterized. There is a long-standing association between the presence of hemolytic *E. coli* and increased severity of urinary tract infection (212). Indeed, HlyA has been associated with hemorrhagic urinary tract infections (213) and was shown to evoke extensive sloughing of bladder epithelium in a mouse model of urinary tract infection (214). HlyA has also been associated with increased acute kidney injury due to GM-CSF induction and subsequent increased pro-inflammatory macrophage activity in the kidney (197). Together these studies establish HlyA as an important virulence factor during UPEC infection; however, its role in NMEC infection has not been explored.

We demonstrated *in vitro* that P2XR-dependent macrophage death is associated with HlyA-expressing NMEC strains (Fig. 23). Furthermore, NMEC 42, the HlyA+ strain that is not visibly hemolytic on blood agar, induces significantly less cell death than other HlyA+ strains further suggesting a relationship between HlyA activity and P2XR activation. This is in line with studies of UPEC which showed that HlyA mediates P2XR activation and cell death in UPEC-infected cells (147). We next demonstrated that P2XR inhibition results in reduced survival and increased bacterial loads following infection of neonatal mice with the HlyA+ NMEC RS218 (Fig. 24A); however, P2XR inhibition had no appreciable effect on the outcome of infection with HlyA- NMEC O18 (Fig. 24B and C). While further studies
are needed to compare the response to other HlyA⁺ and HlyA⁻ NMEC strains in vivo, these data, in combination with data showing increased virulence of NMEC O18 compared to NMEC RS218 (Fig. 16), suggest that HlyA-induced activation of P2XRs serves as an anti-virulence factor and aids in eliciting clearance of the bacteria.

While we have not yet directly assessed the role of HlyA in the activation of NLRP3 during NMEC infection, we did show that NMEC RS218 induces robust IL-1β secretion from macrophages while NMEC O18 does not (Fig 18B). It has also been demonstrated that NLRP3 activation by UPEC isolates is dependent on HlyA expression (113, 114). Therefore, it is likely that NLRP3 activation in response to NMEC is also HlyA-dependent. We found that NLRP3-dependent IL-1 activation and secretion was only protective in NOS2⁻/⁻ neonates (Fig. 15) and we propose this effect was due to the high levels of endogenous NO inhibiting activation of the NLRP3 inflammasome. Interestingly, when we infected NLRP3⁻/⁻ neonatal mice, we did see an apparent increase in bacterial loads compared to what we would expect in wildtype neonates. While we have not directly compared wildtype to NLRP3⁻/⁻ neonates, this observation does appear to be consistent across multiple independent experiments. This may suggest that other downstream effects of NLRP3 activation, such as IL-18 release or GSDMD pore formation, are protective during neonatal NMEC infection even though NLRP3 activation is diminished compared to adults. Although further studies are needed to assess the role of these other NLRP3-dependent mechanisms during neonatal infection, our current
observations suggest that NLRP3 activation could serve as another means by which HlyA elicits a protective response against NMEC infection.

Studies of UPEC infection would suggest that HlyA is an important determinant of UPEC invasion, as evidenced by the isolation of HlyA+ isolates from 31-48% UPEC cystitis cases and over 70% of UPEC pyelonephritis cases (215). In contrast approximately 30% of NMEC isolates express HlyA (20), but this appears to be detrimental to bacterial colonization and lead to improve survival outcomes in a neonatal murine model. While the percent of NMEC isolates expressing HlyA is significantly higher than its frequency in commensal fecal E. coli isolates, our data does suggest that HlyA is not a main determinant of NMEC infection. The propensity of HlyA to be expressed by NMEC isolates could also be due to its presence on large pathogenicity islands (16, 216) containing other virulence factors important to NMEC pathogenicity thereby perpetuating its presence on NMEC isolates. It is possible that HlyA could be one mechanism for initial invasion in NMEC infection, similar to its effect on invasion of renal epithelium during UPEC infection (197); however, it elicits an antimicrobial response against NMEC following initial invasion which results in improved bacterial clearance compared to HlyA− strains. Our i.p. infection technique bypasses the initial invasion step in NMEC infection. It is therefore unclear from our study whether HlyA has a role in early establishment of natural infection. Future studies comparing HlyA+ and HlyA− NMEC, particularly isogenic mutants, using an i.n. infection route could better determine the importance of HlyA in initiating infection.
Working model of macrophage function during NMEC infection

We have observed that depletion of macrophages from neonatal mice prior to infection with NMEC RS218 resulted in increased bacterial loads in all tissues independently of other treatments. Although we have yet not directly compared this effect in a single infection, macrophage depletion has resulted in the highest bacterial loads we have observed for any treatment during NMEC RS218 infection across multiple independent experiments. Collectively, our findings demonstrate multiple potential mechanisms for this macrophage-dependent protection during NMEC infection, all of which are dependent on HlyA secretion by NMEC RS218 (Figure 33). We demonstrate that induction of IL-1β secretion by macrophages is TLR4- and MyD88-dependent (Figs. 4 and 6), likely representing a priming event for both pro-IL-1β and NLRP3 (101). We also show that upregulation of IL-1β mRNA expression was not dependent on HlyA (Fig. 18C); however, secretion of IL-1β, which we found to be NLRP3-dependent (Figs. 7 and 8), occurred only in response to NMEC RS218 (Fig. 18B), suggesting an HlyA-dependent mechanism for NLRP3 activation. While IL-1 signaling was not protective in NMEC RS218-infected wildtype neonates in vivo (Fig. 11), it was protective in the absence of iNOS (Fig. 15), indicating that IL-1 signaling can be protective in the context of NMEC infection. IL-1 is known to have multiple downstream effects during infection which could impart protection. IL-1R signaling activates macrophages to express high levels of MHC-II, as well as secrete high levels of inflammatory cytokines such as IL-6, TNF and IL-1, and enhance phagocytic capacity (185, 217, 218). IL-1R activated macrophages may also have enhanced phagocytic capacity, induce improved antigen presentation to B cells, and promote antibody production (217,
IL-1 signaling via IL-1R also impacts other innate inflammatory cells, as IL-1 serves as a potent neutrophil chemoattractant and mediator of emergency granulopoiesis (219).

Additionally, we see a trend towards increased bacterial loads in NLRP3⁻/⁻ neonates compared to what we expect from wildtype neonates at the same timepoint post-infection. This suggests the possibility for a protective role of other NLRP3-dependent mediators such as GSDMD or IL-18. IL-18 is a pro-inflammatory cytokine with several downstream effects that can promote bacterial clearance during infection. IL-18 promotes production of IFN-γ by T cells and NK cells thereby indirectly increasing the bactericidal activity of macrophages (179, 220). IL-18, like IL-1β, can also enhance production of inflammatory cytokines and help initiate neutrophil recruitment and activation (179, 220). In addition to its role in pyroptosis and cytokine secretion, activated GSDMD can also insert into the mitochondrial membrane, leading to production and release of mitochondrial reactive oxygen species (mROS), which may aid in bactericidal activity (221, 222). NLRP3 has also been reported to have important functions independent of its role in inflammasome activation (223-227). These include production of IFN-γ and mROS (225, 227), as well as increasing protecting epithelial barrier integrity (226). Further studies are needed to determine which of these pathways may be involved in IL-1β-independent NLRP3-mediated protection during NMEC infection.

Finally, we also demonstrate that P2XR-dependent protection during NMEC RS218 infection requires macrophages (Fig. 31). This macrophage-dependent response may be due to a role of P2XR in inducing migration of
monocytes/macrophages to the site of infection, or due to direct activation of macrophages allowing for an improved bactericidal response. We did not identify a robust P2XR-dependent effect on either bacterial uptake or intracellular bacterial killing by peritoneal macrophages (Fig. 32) suggesting that direct impact on these functions is likely not the mechanism by which P2XR signaling is protective. However, we do see P2XR-dependent cell death *in vitro* (Fig. 21) which indicates a direct effect for P2XR signaling on macrophages during NMEC RS218 infection. P2XR signaling could also be working on macrophages indirectly by inducing migration to the site of infection. We observed a decrease in MCP-1 in the peritoneum of neonatal mice treated with BBG, a P2XR inhibitor, (Fig. 29F) which may suggest a role for P2XR signaling in stimulating monocyte chemotaxis. We also noted that IL-1β and IL-6 were diminished in BBG-treated mice (Fig. 29B and C). While we did not see a direct impact of P2XR inhibition on secretion of IL-1β by macrophages *in vitro*, we have shown that both IL-1β and IL-6 are robustly induced by NMEC RS218 infection of macrophages (Fig. 18). Therefore, decreased concentrations of these cytokines in BBG-treated mice may indicate reduced numbers of macrophages/monocytes in the peritoneum and could suggest that inhibition of P2XRs attenuates monocyte/macrophage chemotaxis in response to NMEC RS218.
Figure 33. Working model of macrophage function during NMEC infection.
During NMEC infection, the macrophage response is dependent on HlyA expression. Both NMEC O18 and NMEC RS218 induce transcription of pro-IL-1β via TLR4. Only the HlyA+ strain NMEC RS218 activates NLRP3 leading to IL-1β, IL-1α, and IL-18 secretion. IL-1 signaling can lead to improved bacterial clearance and survival; however, in neonates, high NO blocks NLRP3 inflammasome activation, limiting IL-1 secretion. HlyA pore formation also activates P2XRs. *In vitro*, P2XR activation results in caspase-independent macrophage lysis. *In vivo*, P2XR activation induces macrophage-dependent protection.
Potential effects on neurodevelopment

One ongoing concern in the treatment of neonatal meningitis is the propensity for affected infants to develop long-term neurologic sequelae even in the face of otherwise successful antibiotic treatment. To date, there are no approved therapeutics to limit neurologic damage during neonatal infection, and more research is needed to understand how to balance the elements of the immune response to maximize bacterial clearance while limiting tissue damage.

We provide evidence here that IL-1 is important for bacterial clearance and improved survival in the context of *E. coli* K1 meningitis in adult mice (Fig. 10), and under certain conditions in neonates (Fig. 11). However, overzealous IL-1 activity in the brain is also associated with neuropathology and long-term behavioral alterations. While IL-1 signaling was found to be protective during meningitis induced by *S. pneumoniae* (105) or *S. agalactiae* (104), it has been shown that IL-1R blockade following induction of *S. pneumoniae* meningitis in rats prevented cognitive impairment (107). Furthermore, systemic induction of IL-1 in response to polymicrobial sepsis has been suggested to lead to long-term neurologic alterations without direct infection of the brain (108). While one study found neonatal *E. coli* K1 infection led to learning disabilities (228), to our knowledge, there have not been any studies directly assessing the role of IL-1 in behavioral dysfunction induced by *E. coli* K1 infection. However, as IL-1 signaling can promote neuropathology in other forms of meningitis, treatment strategies that augment the IL-1 response during *E. coli* K1 infection must balance the protective antibacterial effects of IL-1 with potentially detrimental effects on neurodevelopment. IL-1 signaling is highly pleiotropic (102). Therefore,
identification and inhibition of specific detrimental downstream pathways could improve neurologic outcomes while still allowing for the protective antibacterial effects. For example, lithium treatment of neural progenitor cells is able to ameliorate the effects of IL-1β on cell proliferation and differentiation by inhibiting IL-1β-mediated increases in GSK-3β activity (229). Further, in multiple models of S. pneumoniae infection, treatment with lithium prevented hippocampal apoptosis and improved memory outcomes of infected rats (230, 231). IL-1 signaling has also been shown to interfere with the endogenous neuroprotective brain-derived neurotrophic factor (BDNF) signaling pathway (232-234). Treatment with BDNF has already been proposed as an adjunct treatment during meningitis to improve neurologic outcomes (235). Therefore, incorporation of BDNF treatment with therapies that augment IL-1 responses during meningitis may serve to limit deleterious effects of IL-1 on the brain while maintaining its role in bacterial clearance.

P2XRs are extremely important for normal signal transduction within the CNS and brain development (126-128, 236). Therefore, it is not surprising that they are present on many cell types throughout the CNS. It has also been reported that P2X7R expression on macrophages is similar in neonates and adults (109). Nevertheless, neonates are known to have higher concentrations of ectonucleotidases in their blood which breakdown ATP to the anti-inflammatory adenosine (145). Even so, our data demonstrating a deleterious effect of P2XR inhibition on bacterial clearance (Fig.24A) suggests that the massive release of ATP during infection can overwhelm this ectonucleotidase activity. Interestingly, we also found
that P2XR signaling was deleterious to bacterial clearance from the brain when macrophages were depleted from the peritoneum (Fig. 31A). It remains to be seen whether this is a biproduct of overall increased bacterial loads, or if it is indicative of a site- and/or cell-type specific response by P2XR signaling to NMEC RS218 infection. It is also unclear whether increased ATP release within the CNS during meningitis contributes to exacerbated tissue damage or alters the normal physiologic function of neuronal P2XRs in ways that may be deleterious over time. Da Silva et. al. assessed the role of P2XR activation on brain development during systemic inflammation found that while there was no apparent effect on behavioral outcomes, neonatal rats treated with BBG prior to LPS challenge had decreased evidence of oxidative damage in the hippocampus as adults, suggesting that P2XR activation could contribute to long-term neurologic damage (146). Based on this observation and the increased survival rate among BBG-treated rats, it is possible that P2XR inhibition during neonatal infection may prevent neurological mal-effects. However, there are some clear limitations to this study, not the least of which being the use of LPS as a model for live infectious agents. In our infection model we observed reduced survival among BBG-treated mice which makes therapies designed to block P2X signaling less desirable. It is also difficult to interpret the behavioral data, as there were no differences between control animals and LPS-treated animals independently of other treatments. Therefore, it is difficult to determine whether the decrease in oxidative stress markers had any physiologic relevance. Interestingly, Feng et. al., assessed the role of P2X7R on collagen-induced hemorrhage, another non-infectious model of CNS inflammation, using
adult mice as further evidence that P2XR activation is involved in inflammation-induced brain injury and neurologic impairment (237). They found that P2XR signaling mediated NLRP3 inflammasome activation as well as secretion of IL-1β and IL-18 and concluded that it was this NLRP3 response which ultimately induced the neurologic damage (237). Since we determined that NLRP3 activation was independent of P2XR signaling during NMEC RS218 infection, it is unclear what effect P2XR activation may have on neurologic function during or following NMEC RS218 infection.

**NO-inhibition as a potential mechanism for improving neonatal vaccine efficacy**

In addition to providing direct early protection against invading pathogens, the innate immune system also serves to inform and activate the adaptive immune response via direct interactions and cytokine production (238). Therefore, alterations in the neonatal innate immune response could in part explain diminished T and B cell activation and memory formation, which have limited the effective production of vaccines for use in neonatal populations. NLPR3 inflammasome activity has been implicated as an important component of the immune stimulatory effects of alum (167). Both IL-1 and IL-18 are important cytokines for induction of T H1 and T H17 responses (238, 239) and adjuvants that target NLRP3 activation have been suggested for use in future vaccine strategies (238, 239). Particularly, NLRP3-activating adjuvants were proposed for use in vaccines aimed at common bacterial respiratory pathogens of early childhood, including *S. pneumoniae* (239). Therefore, deficiencies in NLRP3 activation in neonates could serve as a factor in the diminished responsiveness of neonates,
and particularly of premature infants to vaccination. Our findings that NO inhibition serves to enhance secretion of bioactive IL-1 in response to infection may also have implications in the design of vaccines for use in neonates. Potentially, administration of iNOS inhibitors in conjunction with vaccine administration could improve NLRP3 responses and better elicit adaptive cell activation and the formation of a protective memory response.

**Improving diagnostic and prognostic procedures**

Early diagnosis and initiation of appropriate antibiotic treatment is key to prevention of adverse outcomes associated with neonatal sepsis and meningitis (22, 39). Unfortunately, several limitations currently hinder the early recognition and diagnosis of these infections. Early clinical signs of sepsis and meningitis are often non-specific and overlap significantly with non-infectious conditions of neonates (21-23). The current gold standard of culturing the blood and/or CSF requires prolonged wait times for cultures to grow and may be complicated by initiation of antibiotics prior to culture as well as varied infection kinetics (22, 23). PCR-based diagnostics are a potential improvement to culture-based methods due to decreased run times and increased sensitivity. Our data would suggest that the addition of PCR panels designed to assess the presence of specific virulence genes, such as hlyA, may provide important prognostic information which could guide therapeutic decision-making.

While various biomarkers have been suggested for quick identification of sepsis in infants, no single test has been able to reliably diagnose infection (240). Metabolomic profiling is an up-and-coming area of study and has been suggested
as a potential early diagnostic tool in the recognition of neonatal sepsis (240, 241). Although relatively few studies have assessed the use of metabolomics as a marker of neonatal infection, the studies which have been done show promise in the ability of metabolomics data to not only diagnose infection and offer prognostic indicators, but to also help distinguish viral infection from bacterial etiologies (241-244). The data presented here could support the use of metabolomic analyses in the differentiation of NMEC isolates which could serve as prognostic indicators as well as guide potential treatment plans. P2XR activation, which we found to be associated with HlyA expression and decreased NMEC virulence, plays a key role in modulation of lipid metabolism and eicosanoid production (245) as well as upregulation of glycolysis and GLUT-1 expression (246). Therefore, based on the data presented here, future research in our lab specifically exploring the metabolomic profiles of neonatal mice infected with HlyA+ and HlyA- NMEC strains could uncover key temporal metabolic biomarkers that distinguish between NMEC strains that vary in virulence.

**Conclusion**

We found that IL-1 signaling was protective during NMEC RS218 infection of adult mice. In contrast, IL-1 confers protection in neonatal murine infection only in the absence of iNOS. This difference is likely attributable to the enhanced iNOS activity in neonates coupled with the ability of NO to block NLRP3 activation. We also present data suggesting that HlyA expression by NMEC may serve as an anti-virulence factor via activation of P2XRs resulting in macrophage-dependent bacterial clearance. Together, the data presented here demonstrates the
importance of both host age and bacterial virulence factors to the innate immune response to NMEC infection. While more research is needed to fully define the mechanisms of these interactions, our data reinforce the need for continued exploration into the innate immune response to NMEC infection. These data further serve to highlight the importance of using age-relevant host models and multiple NMEC strains in future studies focused on development of potential diagnostic, prognostic, and treatment tools.
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VITA

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