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Role of Toll-like receptor 2 in innate resistance to group B Streptococcus

Iain R. Asplin[a], **David J. Carl**[b], **Sing Sing Way**[b], and **Amanda L Jones**[b,*]

a *Division of Critical Care Medicine, Children's Hospital and, Regional Medical Center and Department of Pediatrics, University of Washington, Seattle, WA 98105, USA*

b *Division of Infectious Disease, Children's Hospital and, Regional Medical Center and Department of Pediatrics, University of Washington, Seattle, WA 98105, USA*

Abstract

The Gram-positive bacterium Group B Streptococcus (GBS) is an important cause of serious neonatal and adult infections. Toll-like receptor 2 (TLR2) recognizes components of the cell wall of Gram-positive bacteria and is critical for defense against certain invasive pathogens. In GBS, penicillin-binding protein 1a (PBP1a), encoded by *ponA*, is required for virulence. PBPs participate in cell wall synthesis and in previous studies; the absence of PBP1a was shown to result in subtle changes in the cell wall ultrastructure. Here, we examine the role of TLR2 in defense against GBS infection and the impact of mutation of *ponA* on TLR2-mediated host responses. We demonstrate TLR2-recognition of both WT GBS and the *ponA* mutant *in vitro*. TLR2$^{-/-}$ mice were significantly more susceptible than WT mice to infection with either strain of GBS, indicating a crucial role for TLR2 in defense against GBS. Additionally, the *ponA* mutant was severely attenuated for virulence in both strains of mice. The mutation in *ponA* did not affect cytokine expression by WT or TLR2$^{-/-}$ mice. These data indicate that TLR2 is required for host defense against GBS and this response is unaffected by the absence of PBP1a and the resultant changes in cell wall ultrastructure.

Keywords

Streptococcus agalactiae; Toll-like receptor 2; Penicillin-binding protein 1A

1. Introduction

The Gram-positive bacterium Group B Streptococcus (GBS, *Streptococcus agalactiae*) is an important cause of neonatal sepsis, pneumonia, and meningitis and is increasingly implicated in soft-tissue and invasive infections in the elderly and the immunocompromised [1–6]. Neonatal infections result from ascending movement of GBS from the lower maternal genital tract into the amniotic fluid with subsequent aspiration prior to birth, or from aspiration of infected maternal secretions during delivery. GBS then invades across epithelial and endothelial layers in the neonatal lung to cause bacteremia [7]. Accordingly, host defense against invasive GBS infection requires either containment to prevent invasion and subsequent bloodstream infection, or rapid elimination once such bacteremia occurs.

*Corresponding author: Amanda L. Jones, Children's Hospital and Regional Medical Center, 307 Westlake Ave N, Suite 300, Seattle, WA 98109, USA, Phone +1 206 987 3573, Fax +1 206 987 7311, Email amanda.jones@seattlechildrens.org.

Neonates are relatively deficient in various aspects of adaptive immunity [8]. Therefore, neonates are dependant upon the innate immune system in order to mount a successful immune response to invading pathogens, such as GBS [8]. Activation of the innate immune response, in neonates and in adults, is controlled in large part by the Toll-like receptor (TLR) family of pattern-recognition receptors. TLRs are transmembrane proteins that recognize specific pathogen-associated molecular patterns. TLRs activate immune responses through several intracellular signaling proteins, such as MyD88, Toll-/interleukin-1-receptor (TIR) domain containing adaptor-inducing interferon (TRIF), TRIF-related adapter molecule (TRAM), and TIR domain containing adapter protein (TIRAP) [9,10]. Signaling through these adapter proteins results in cell activation, induction of co-stimulatory proteins, and production of pro-inflammatory cytokines, such as TNF-α and IL-6 [10,11], and other proteins important for host defense, such as antimicrobial peptides (AMP) [12].

GBS and other Gram-positive bacteria can activate host immune effector cells through TLR2 [13,14]. The specific component or components of these bacteria that can initiate intra-cellular signaling cascades through TLR2 has not been conclusively identified. Lipopeptides and peptidoglycan (PG) from Gram-positive and Gram-negative bacteria, and lipoteichoic acid (LTA) from Gram-positive bacteria have all been proposed as the ligand(s) for TLR2 [11,12, 15–18]. How TLR2 recognizes GBS is not completely elucidated. TLR2, as part of a heterodimer with TLR6, can bind the LTA of GBS [19] and may recognize other components of the cell wall of Gram-positive bacteria. Henneke *et al.* have demonstrated that TLR2 recognizes soluble secreted factors from GBS, in addition to LTA [20].

We have previously shown that PBP1a, a surface-localized penicillin-binding protein encoded by *ponA*, plays an important role in the pathogenesis of GBS infection as a mutant lacking PBP1a is attenuated for virulence in a neonatal rat sepsis infection model [21,22] and cleared more rapidly from the lungs of neonatal rat pups in an aerosol infection model [23]. Additional characterization of the mutant revealed that PBP1a promotes resistance of GBS to killing by host innate immune antimicrobial peptides, which likely contributes to the virulence defect of this mutant [24].

PBP1a is a transmembrane bifunctional PG synthase that catalyzes the oligomerization of nascent PG strands and the cross-linking of these PG chains into the mature cell wall [25]. Orthologs of PBP1a are found in both Gram-positive and Gram-negative bacteria [26–28]. The absence of PBP1a results in subtle changes in the cell wall ultrastructure including a reduced cross-linking of the PG that is detectable by HPLC analysis [24]. As TLR2 recognizes the cell wall of Gram-positive bacteria, in this study we investigate whether the mutation in PBP1a affects recognition of GBS by TLR2 and examine the contribution of TLR2 to innate immune defense against infection by GBS.

2. Results

2.1. TLR2-mediated cell activation by WT GBS and the ponA mutant

Although GBS can activate cells through TLR2, the specific moiety or moieties responsible for this activation have not been identified [19,20,29,30]. We compared the *ponA* mutant with WT GBS in TLR2 gain-of-function assays to examine whether TLR2-mediated cell activation differed between the two strains. In these experiments, the activation of HeLa cells, transiently transfected with either human or murine TLR2, after stimulation with varying concentrations of WT GBS or the *ponA* mutant, was measured using a NF-κB luciferase reporter system.

Serial dilutions of either WT GBS or the *ponA* mutant GBS were added to HeLa cells transfected with either human TLR2 or murine TLR2. A dose-dependent activation of NF-κB mediated by both human and murine TLR2 was observed (Fig. 1). At an inoculum of 5 ×

10^6 CFU, activation of NF-κB by TLR2 was significantly increased by exposure to WT GBS, when compared to non-transfected HeLa cells. The same inoculum of the *ponA* mutant also activated NF-κB to an equivalent extent to that of WT GBS. This stimulation of NF-κB activation was still evident for both strains of GBS at an inoculum of 2.5×10^6 CFU, but was not detectable at 1.25×10^6 CFU.

No increase in luminescence was seen without infecting the HeLa cells with GBS (Fig. 1, no stimulation). The responsiveness of the transfected HeLa cells to a TLR2 agonist was confirmed with the use of heat-killed flagellin-deficient *Listeria monocytogenes* [31] (data not shown). A robust luminescence was seen following exposure of transfected HeLa cells with IL-1, which stimulates NF-κB activation independently of TLR2 (Fig. 1, IL-1) [32,33].

2.2. Virulence of WT GBS and the ponA mutant in WT adult mice

The *ponA* mutant was originally identified in a virulence screen in an intraperitoneal sepsis model in neonatal rat pups. In this model, the LD_{50} of the *ponA* mutant was 10 to 80-fold higher than WT GBS [22]. To determine whether the *ponA* mutant was also attenuated for virulence in adult mice, we compared the virulence of the isogenic strains in a mouse sepsis infection model, as described in section 5.4. The results from two independent determinations of the LD_{50} of WT GBS and the *ponA* mutant in WT mice were combined, as described in section 5.7. The LD_{50} for WT GBS was 3.6×10^7 CFU (95% confidence interval $1.6 \times 10^7 - 8.2 \times 10^8$ CFU), as compared to a LD_{50} for the *ponA* mutant of 3.6×10^8 CFU (95% confidence interval $7.8 \times 10^7 - 1.6 \times 10^9$ CFU), a statistically significant difference ($p = 0.01$) in virulence, similar to our previous studies in neonatal rat pups [22].

2.3. Time to death of WT mice following infection with isogenic GBS strains

To investigate any difference in kinetics of killing between WT GBS and the *ponA* mutant, we performed time to death assays in WT mice. A range of inocula were used and a representative experiment in which the mice were infected with ~2.0×10^8 CFU of WT or *ponA* mutant GBS is shown in Fig. 2. More mice succumbed to infection by the WT strain than to the *ponA* mutant, confirming the results of our LD_{50} assays; however, no change in the rapidity of killing was noted between the two strains of GBS.

2.4. Competitive Index Assays in WT mice

To investigate how the isogenic GBS strains competed in a mixed infection, we performed competitive index assays. Following 18–24 hours of infection, all mice were bacteremic. However, very few to none of the *ponA* mutant were recovered from WT mice (Table 1). The competitive index was $<0.01 \pm 0.01$ in WT mice, consistent with severe attenuation of the *ponA* mutant compared to the WT strain that we observed in the LD_{50} and time to death assays. These data indicated that the presence of the WT strain does not complement the virulence defect of the *ponA* mutant in a mixed infection.

2.5. Time to death of $TLR2^{-/-}$ mice following infection with the isogenic GBS strains

TLR2 recognizes components of the cell wall of Gram-positive bacteria and is, therefore, an important component of the innate immune response to Gram-positive bacterial infection. We employed $TLR2^{-/-}$ mice to investigate whether TLR2 enables mice to differentially respond to WT GBS, which expresses PBP1a, compared to the *ponA* mutant, which does not. We first addressed this question by examining the impact of the mutation in *ponA* in time to death assays using $TLR2^{-/-}$ mice. Similar to the studies described above, the $TLR2^{-/-}$ mice were infected with a range of inocula. A representative experiment in which the mice were infected with ~2.0×10^8 CFU of WT or *ponA* mutant GBS is shown in Fig. 3. The $TLR2^{-/-}$ mice were more

susceptible to infection by WT GBS than to the *ponA* mutant and the kinetics of killing of the TLR2$^{-/-}$ mice did not appear to differ between the two strains of GBS.

2.6. Competitive Index Assays in TLR2$^{-/-}$ mice

To further investigate the role of TLR2 in the response to GBS, we performed competitive index assays in TLR2$^{-/-}$ mice. As with the WT mice, following inoculation with a 1:1 ratio of WT and *ponA* mutant GBS, by 18–24 hours following inoculation all of the TLR2$^{-/-}$ mice were bacteremic. However, very few to none of the *ponA* mutant were recovered from the TLR2$^{-/-}$ mice and the competitive index was calculated to be <0.007 ± 0.01 (Table 1). This result was consistent with severe attenuation of the *ponA* mutant compared to the WT strain of GBS in TLR2$^{-/-}$ mice in the time to death assays and indicates that the presence of the WT strain does not complement the virulence defect of the *ponA* in TLR2$^{-/-}$ mice. The competitive index determined in the TLR2$^{-/-}$ mice was not significantly different from that determined in WT mice ($p = 0.56$).

2.7. Induction of cytokine production by WT GBS and the ponA mutant in WT and TLR2$^{-/}$ mice

Binding of TLR2 to its ligand(s) activates intracellular signaling cascades, stimulates activation of NF-κB, and results in the production of pro-inflammatory cytokines, such as TNF-α and IL-6 [10,11]. As TLR2-mediated NF-κB production increased *in vitro* following infection with both WT GBS and the *ponA* mutant (see Fig. 1), we were interested to determine whether production of TNF-α and IL-6 in WT mice differed following infection with the two strains of GBS. In addition, we wondered whether the increased mortality seen in TLR2$^{-/-}$ mice following infection with either WT GBS or the *ponA* mutant might result from inability to mount an effective inflammatory response following infection. To investigate these questions, we measured the amount of TNF-α and IL-6 produced *in vivo* following infection of WT and TLR2$^{-/-}$ mice by either WT GBS or the *ponA* mutant.

Blood was collected from WT mice following infection with either WT GBS or the *ponA* mutant. The bacterial load in the blood, serum TNF-α and IL-6 levels were determined for each mouse. As shown in Fig. 4A, for a given bacterial load, WT mice produced the same amount of TNF-α regardless of the GBS strain that was administered Similar results were obtained using TLR2$^{-/-}$ mice where following infection with an equivalent bacterial load of either strain; we detected the same amount of TNF-α produced (Fig. 4B).

We also quantified the amount of IL-6 produced in response to infection. The WT mice produced the same amount of IL-6 for the same bacterial load of either of the isogenic GBS strains (Fig. 4C). A similar observation was made for IL-6 production by TLR2$^{-/-}$ mice with equivalent bacterial loads of the WT and *ponA* mutant strain (Fig. 4D). These data show that both WT and TLR2$^{-/-}$ mice are able to generate an equivalent robust TNF-α and IL-6 response following infection with either WT GBS or the *ponA* mutant. Linear regression modeling demonstrated that the amount of TNF-α or IL-6 produced was correlated with the level of bacteremia; the amount of TNF-α or IL-6 produced was dependent only upon the CFU of bacteria present in the blood of the infected mice, rather than the strain of GBS that was used.

3. Discussion

GBS is a common commensal organism in the female vaginal tract, where it is held in check by as yet unknown mechanisms [34,35]. When GBS comes in contact with epithelial or mucosal surfaces in a relatively immunocompromised host, such as a neonate or an elderly or diabetic adult, it has the capacity to mount an invasive infection. Neonates at risk for GBS infection lack effective adaptive immune responses and are dependent upon components of the

innate immune system, such as the TLRs, for defense [8]. Characterizing the interaction of GBS with components of innate immunity enhances our understanding of the pathogenesis of neonatal invasive disease.

TLR2 is activated by cell-wall components of Gram-positive bacteria, such as GBS, released by the bacterium or as an integral part of its cell wall. TLR2 plays an important role in host defense against Gram-positive organisms and its role in defense against GBS is the subject of ongoing investigation [19,20,36–38]. TLRs such as TLR2, may not bind directly to their target. Instead, they may interact with other pattern recognition receptors which do bind the target and serve as a scaffold for the subsequent intracellular signaling. This would allow a given TLR to be able to potentially recognize and respond to several pathogen-associated molecular patterns [39].

The integrity of the bacterial cell wall is essential for bacterial survival. Biosynthesis of cell wall PG is the function of PBPs. Class A PBPs such as GBS PBP1a are bifunctional proteins with both transglycosylase activity that extends the cell wall glycan chains and a transpeptidase activity that cross-links the PG chains [25]. There appears to be functional redundancy in PBP activity such that inactivation of one PBP can often be compensated for by others [25–27,40–43]. We have previously shown that PBP1a is important for virulence of GBS in neonatal rat pups, both in an intraperitoneal sepsis model [21,22] and in lung colonization model [23].

HPLC analysis of PG from the GBS *ponA* mutant revealed subtle alterations in the cell wall compared to the WT strain. Specifically, a reduction in the degree of cross-linking of the PG was detected [24]. As TLR2 is known to recognize components the cell wall of Gram-positive bacteria, we investigated whether recognition of GBS was affected by the absence of PBP1a and the contribution of TLR2 to innate immune defense against invasive GBS infection.

In an *in vitro* transfection system, we were able to reconstitute the TLR2 signaling pathway and demonstrate that both WT GBS and the *ponA* mutant are recognized by TLR2 and activate signaling in a dose-dependent manner. When using equivalent numbers of each strain, we did not detect any difference in the magnitude of activation, suggesting that the absence of PBP1a and the resultant changes in the cell wall do not affect recognition by TLR2 or subsequent intracellular signaling.

We then compared the virulence of the isogenic GBS strains in WT mice in a sepsis infection model. The *ponA* mutant was significantly attenuated for virulence compared to WT GBS as measured by the LD_{50} assays; the LD_{50} for the mutant was 10-fold greater than the WT strain. The *ponA* mutant also displayed a competition defect in our competitive index assays using WT mice. This suggests that the survival defect of the *ponA* mutant is not due to lack of production of a secreted factor, such as a toxin as in a mixed infection, as such a deficient factor would be provided by the WT GBS. These data support our hypothesis that the survival defect of the *ponA* mutant is linked to the changes in the cell wall. In time to death experiments, for a given dose of bacteria, fewer mice succumbed to infection with the *ponA* mutant than to the WT strain. There did not appear to be a difference in the kinetics of killing between the two strains of GBS. These data suggest that the virulence defect of the *ponA* mutant is not simply due to a delayed ability of the *ponA* mutant to mount an invasive infection, as the time course of when deaths occurred was similar between the two strains. Rather, this would imply that WT mice are more likely to clear the *ponA* mutant, but, if unable to do so, are susceptible to lethal infection.

We then examined whether TLR2-mediated recognition was altered by the absence of PBP1a and investigated the contribution of TLR2 to protecting mice from lethal infection. Our hypothesis was that if PBP1a was required for TLR2-mediated immune recognition and defense, then the attenuation of the *ponA* mutant that we observed in WT mice would be

ameliorated or eliminated in TLR2$^{-/-}$ mice. In our studies, the TLR2$^{-/-}$ mice were more susceptible than WT mice to both the WT and *ponA* mutant. These observations are consistent with studies demonstrating an increase in susceptibility to WT GBS infection [37] and increased mortality in the absence of TLR2 following infection with other Gram-positive bacteria [44]. Additionally, there was also no difference in the kinetics of killing by the two strains in the TLR2$^{-/-}$ mice. Consistent with our *in vitro* observations, these data indicated that the absence of PBP1a does affect TLR2-mediated recognition of GBS *in vivo*.

To further define the role of TLR2 in the response to GBS infection, we investigated the ability of WT and TLR2$^{-/-}$ mice to mount a pro-inflammatory cytokine response. Interestingly, both WT and TLR2$^{-/-}$ mice produced similar amounts of TNF-α and IL-6 when bacteremic with either the WT or *ponA* mutant GBS strain. This suggests that TLR2$^{-/-}$ mice are able to generate an inflammatory response, as measured by TNF-α and IL-6 production, equivalent to that of WT mice following infection with GBS despite lacking this important component of the innate immune system. Our studies indicate that TLR2 is important for defense against GBS infection as TLR2$^{-/-}$ mice are more susceptible to infection with both the WT strain and *ponA* mutant. However, TLR2$^{-/-}$ mice mounted a robust cytokine response that was essentially equivalent to that of WT mice suggesting that other mechanisms of cytokine production that are TLR2-independent are operating. Such a mechanism(s) would appear to be less effective without TLR2, based on time to death assays. Further studies are needed to define the mechanisms by which TNF-α and IL-6 are produced in response to GBS infection in a TLR2$^{-/-}$ background.

4. Conclusions

Our data demonstrate an important role for TLR2 in defense against GBS infection. The absence of TLR2 profoundly impaired the ability of mice to survive infection by GBS but did not significantly alter early, pro-inflammatory responses. TLR2 did not appear to mediate the early virulence defect of the *ponA* mutant. However, the absence of PBP1a clearly results in profound attenuation of GBS virulence, a phenotype we have now extended to healthy, adult WT mice. Additionally, these studies demonstrate that that the absence of PBP1a does not result in any changes to the cell wall of GBS that impact effective recognition by TLR2. This would suggest that TLR2 and the innate immune system is tolerant to minor changes in their pathogen-associated molecular targets such as the cell wall, which might otherwise prevent recognition and the generation of an effective innate immune response.

5. Materials and Methods

5.1 Bacterial strains

The WT GBS strain is A909, a serotype Ia clinical isolate [45,46]. The *ponA* mutant, AJ3F6, is derived from A909 and does not produce PBP1a due to a Tn*917* transposon insertion in the *ponA* gene [21]. Tn*917* contains an erythromycin resistance gene which allows for selection of transposon-containing GBS. This transposon is stably maintained even in the absence of erythromycin (Em) and does not have polar effects on the expression of adjacent genes [21, 22]. Production of well-studied virulence factors such as capsular polysaccharide and hemolysin are unaffected in AJ3F6 [21,22]. Bacteria were grown in tryptic soy broth (TSB; Difco, Franklin Lakes, NJ) or on tryptic soy agar (TSA) at 37°C in 5% CO_2. Prior to use, the bacteria were grown overnight, sub-cultured to mid-log phase, washed, resuspended in PBS and adjusted to the appropriate concentration.

5.2 Transfection assays

HeLa cells were maintained in Dulbecco's modified Eagle's medium (GibcoBRL, Rockville, MD) with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT). The NF-κB reporter

construct (ELAM-1 firefly luciferase, ELAM-Luc), the β-actin-*Renilla* luciferase reporter construct (β-actin *Renilla*-Luc), the modified pDisplay expression vector, and the expression constructs for murine TLR2 (pmuTLR2) and human TLR2 (phuTLR2) have been described previously [47,48]. HeLa cells (4.0×10^4) were seeded into 96-well plates and transfected using Polyfect reagent (Qiagen, Valencia, CA) with the following amounts of DNA per well: ELAM-Luc, 0.03 μg; β-actin *Renilla*-Luc, 0.009 μg; and full-length human or murine TLR2 (minus the signal sequence) cloned in frame with the signal sequence in pDisplay (Invitrogen, Carlsbad, CA), 0.03 μg. The total amount of DNA per well was adjusted to 0.30 μg with empty pDisplay vector. Following transfection, the cells were allowed to grow to confluency and, twenty-four hours after transfection, the cells were stimulated with either WT GBS or the *ponA* mutant for 2 hours. Heat-killed flagellin-deficient *Listeria monocytogenes* was used as a positive TLR2-ligand control in one experiment. Human IL-1β (10 ng/ml) was used a positive control for production of NF-κB. Transfected HeLa cells were then lysed, and the amounts of Firefly and *Renilla* luciferase light units in the lysates were quantified with the Dual-Luciferase reporter assay system (Promega, Madison, WI), which allows for correction of any variability in transfection efficiency. Expression of NF-κB (relative light units) is represented as the ratio of Firefly (ELAM-Luc) to *Renilla* (β-actin *Renilla*-Luc) luciferase values per well.

5.3 Animal studies

WT mice were C57BL/6 mice obtained from Harlan, Indianapolis, IN. $TLR2^{-/-}$ mice backcrossed for 10 generations to C57BL/6 were generated by Dr. Shizuo Akira [11]. All inoculations of bacteria were intraperitoneal and in a volume of 100 μl in PBS. Mice were humanely sacrificed when moribund. All experiments were approved by the Institutional Animal Care and Use Committee.

5.4 LD_{50} Assays and time to death following infection with GBS

To determine the dose of bacteria sufficient to kill 50% of infected mice (LD_{50}), WT mice were infected with serial dilutions (from approximately 2×10^7 CFU to 2×10^{10} CFU) of either WT GBS or the *ponA* mutant. The number of deaths in each group and the number of survivors was used to calculate the LD_{50} at five days. For time to death experiments, WT mice and $TLR2^{-/-}$ mice were infected with either WT GBS or the *ponA* mutant. To permit a comparison of the susceptibility of WT and $TLR2^{-/-}$ mice to killing by WT or *ponA* mutant GBS, all time to death assays were performed in parallel with age-, weight-, and sex-matched WT and $TLR2^{-/-}$ mice using the same preparations of WT GBS and *ponA* mutant GBS. Each group of mice was then followed for five days and the time of death recorded when the mice were moribund and sacrificed. For both LD_{50} and time to death experiments, blood was collected from the mice at the time of death when possible and serial dilutions were plated out to confirm that the animals had succumbed to GBS infection. In some cases, the spleens and livers were harvested, homogenized, and aliquots of this homogenate were serially diluted and plated out to ensure that only GBS was present.

5.5 Competitive Index assays

Competitive index assays were performed as described [22]. Briefly, WT mice and $TLR2^{-/-}$ mice were infected with both WT GBS and the *ponA* mutant in a 1:1 ratio of the two bacterial strains at a combined inoculum of 1×10^8 CFU. Serial dilutions of WT GBS and the *ponA* mutant were plated on TSA to determine the exact inoculum and the exact ratio of WT to *ponA* mutant injected. After sacrifice at 18 – 24 hours following infection, blood from each animal was plated on both TSA plates and plates of TSA containing 1 ug/ml Erythromycin (TSA/Em). The total number of bacteria was determined from the TSA plates, while the number of *ponA* mutant bacteria was determined from the TSA/Em plates. The competitive index is defined as the ratio of mutant to WT bacteria recovered divided by ratio of mutant to WT

bacteria that were inoculated [21,22]. When no *ponA* mutant was recovered, and the competitive index was calculated assuming that one of the *ponA* mutant bacteria had been recovered and the competitive index is expressed as less than this number. For these assays, a competitive index less than 0.1 was considered severe attenuation.

5.6 Cytokine assays

WT or TLR2$^{-/-}$ mice were infected with either WT GBS or the *ponA* mutant. After sacrifice at 18 – 24 hours following infection, serial dilutions of blood were plated out to determine the level of bacteremia. The blood was allowed to clot and the serum concentrations of TNF-α and IL-6 were determined by ELISA (DuoSet® ELISA Development System, R&D Systems, Minneapolis, MN).

5.7 Statistical Analyses

LD_{50} values were estimated via a logistic regression model using Stata statistical software (Stata Statistical Software Release 9, College Station, TX). Data from two separate experiments were combined in the logistic modeling. A two-tailed unpaired Student's t-test was used to assess the statistical significance of the difference in competitive indices determined in WT mice from those determined in TLR2$^{-/-}$ mice. For the cytokine assays, linear regression modeling was performed. Analyses evaluated partial correlations of the type of mouse, strain of bacteria, and the interaction between type of mouse and strain of bacteria. Data from three separate experiments were pooled and used in modeling. SPSS statistical software (SPSS for Windows 10.0, Chicago, IL) was used for these analyses. Statistical significance was set at $p < 0.05$ except where noted.

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Abbreviations

GBS
: Group B Streptococcus

PBP
: Penicillin-binding protein

PG
: Peptidoglycan

WT
: Wild-type

TLR
: Toll-like receptor

TNF
: Tumor necrosis factor

IL-6
: Interleukin-6

AMP
: Antimicrobial peptide

HPLC

High-Performance Liquid Chromatography

Em

Erythromycin

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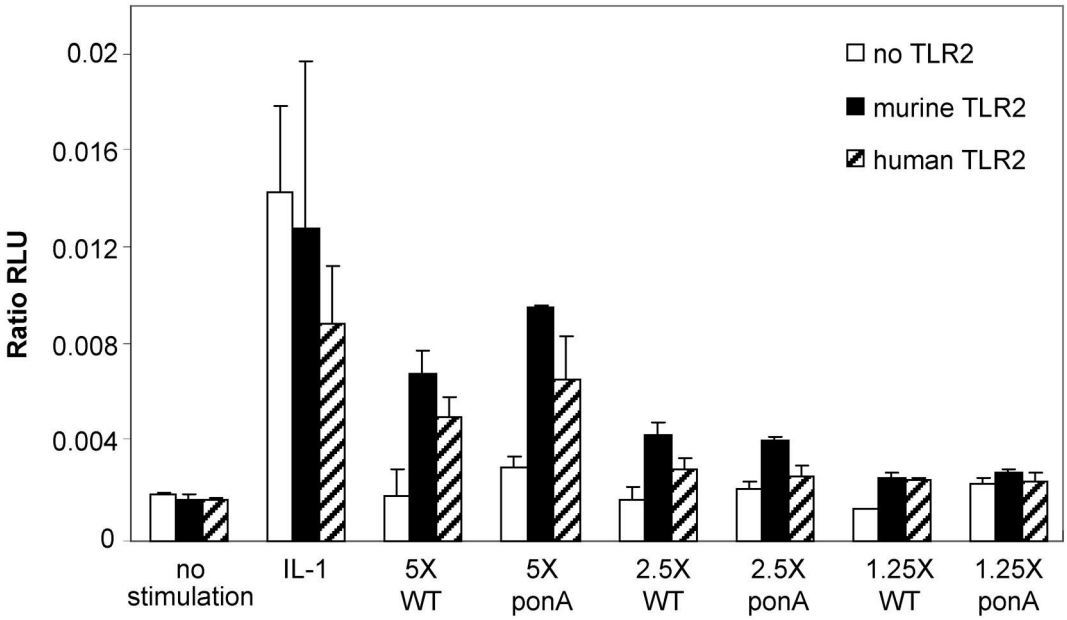

Figure 1.

WT GBS and the *ponA* mutant activate human and murine TLR2. HeLa cells were transiently transfected with human TLR2, murine TLR2, or empty vector. WT GBS (WT) or the *ponA* mutant (*ponA*) at a range of concentrations (X equals 10^6 CFU of bacteria) was added to the HeLa cells and activation of TLR2 measured. No stimulation indicates cells were where only media was added and IL-1 is a positive control that activates NF-κB production in TLR2-independent manner. Data shown are representative of 4 separate experiments performed in duplicate ± SD.

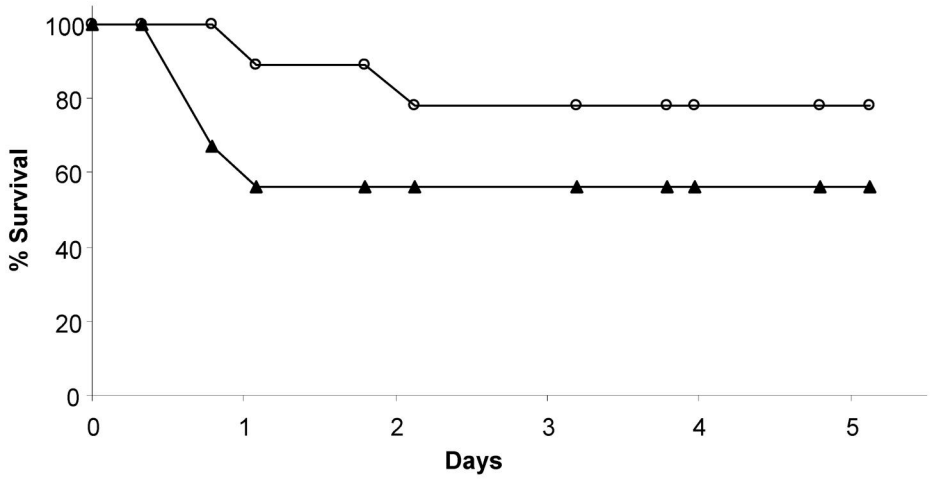

Figure 2.

Time to death assays in WT mice following infection. WT mice were infected with an equivalent dose of either WT GBS (▲; n=9) or *ponA* mutant (○; n=9). The number of mice in each group is indicated in the figure. Data shown are representative of 3 independent experiments.

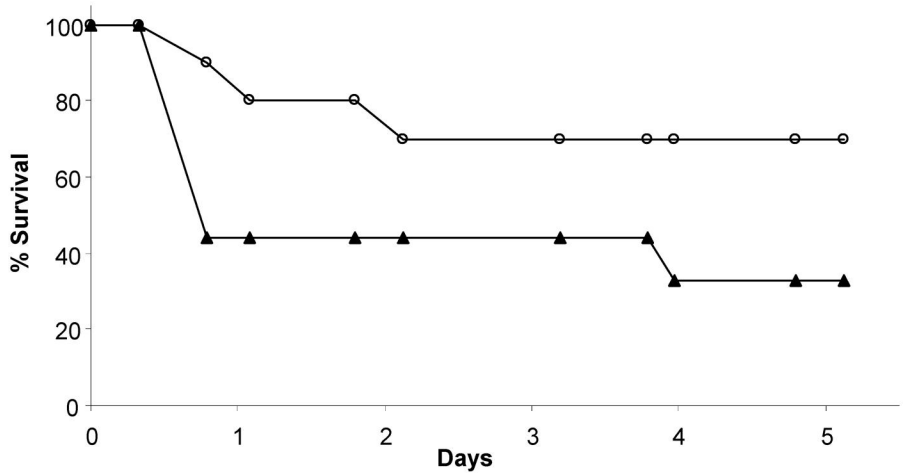

Figure 3.

Time to death in TLR2$^{-/-}$ mice following infection. TLR2$^{-/-}$ mice were infected with an equivalent dose of either WT GBS (▲; n=10) or *ponA* mutant (○; n=9). The number of mice in each group is indicated in the figure. Data shown are representative of 3 independent experiments.

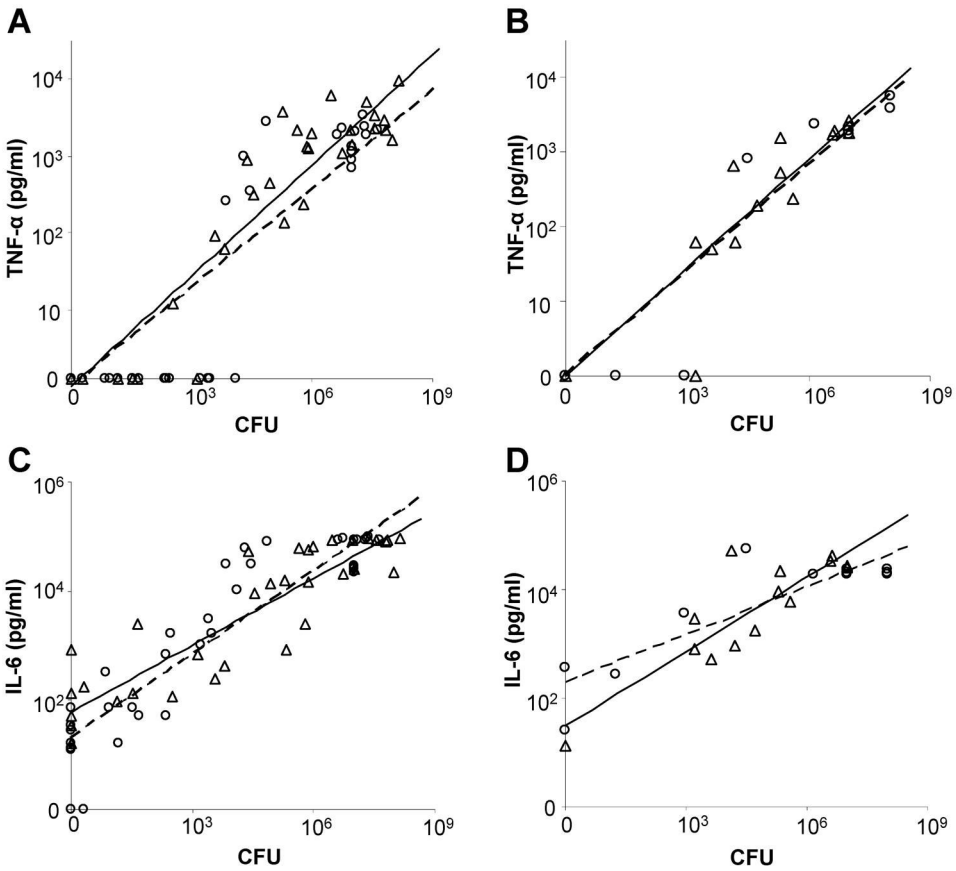

Figure 4.

Cytokine production by WT and TLR2$^{-/-}$ mice following infection with WT and *ponA* mutant GBS. Mice were infected with either WT GBS (▵, solid line) or the *ponA* mutant (○, dashed line). The bacterial load in the blood and levels of TNF-α and IL-6 in the serum were quantified. TNF-α (pg/ml) produced by WT mice per CFU (A) and TLR2$^{-/-}$ mice (B). IL-6 (pg/ml) produced by WT mice per CFU (C) and TLR2$^{-/-}$ mice (D).

Table 1

Competitive Index of WT and *ponA* mutant GBS in WT and TLR2$^{-/-}$ adult mice

Strain of Mouse	Total # of bacteria recovered[a]	# of *ponA* mutant recovered[a]	Competitive Index
WT	28	<1	<0.0328
	149	1	0.00616
	3.39×10^3	<17	<0.00459
	4.85×10^3	<12	<0.0018
	6.70×10^3	201	0.0275
	9.09×10^3	<45	<0.00459
	1.17×10^5	<293	<0.0018
	2.13×10^5	$<1.07 \times 10^3$	<0.00459
	6.08×10^6	$<1.52 \times 10^4$	<0.0018
TLR2$^{-/-}$	12.3	0.9	0.039
	71	0.7	0.00935
	94	1.4	0.0084
	878	4	0.0042
	3.78×10^3	<9	<0.0021
	4.80×10^3	<12	<0.0021
	6.68×10^3	<17	<0.0021
	1.1×10^5	1.10×10^3	0.0056
	1.43×10^5	<715	<0.00467
	6.37×10^5	$<1.59 \times 10^3$	<0.00234
	1.40×10^6	$<7.0 \times 10^3$	<0.00467
	3.80×10^6	$<1.9 \times 10^4$	<0.00467
	4.26×10^6	$<2.13 \times 10^4$	<0.00467
	4.84×10^6	$<1.21 \times 10^4$	<0.0021

[a] CFU/10 μl of blood