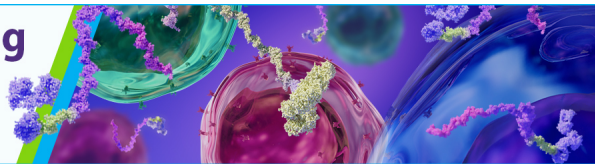


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Dual Role of TLR2 and Myeloid Differentiation Factor 88 in a Mouse Model of Invasive Group B Streptococcal Disease¹

Giuseppe Mancuso,* Angelina Midiri,* Concetta Beninati,* Carmelo Biondo,* Roberta Galbo,* Shizuo Akira,[†] Philipp Henneke,^{‡§} Douglas Golenbock,[‡] and Giuseppe Teti^{2*}

Toll-like receptors (TLRs) are involved in pathogen recognition by the innate immune system. Different TLRs and the adaptor molecule myeloid differentiation factor 88 (MyD88) were previously shown to mediate in vitro cell activation induced by group B streptococcus (GBS). The present study examined the potential in vivo roles of TLR2 and MyD88 during infection with GBS. When pups were infected locally with a low bacterial dose, none of the TLR2- or MyD88-deficient mice, but all of the wild-type ones, were able to prevent systemic spread of GBS from the initial focus. Bacterial burden was higher in MyD88- than in TLR2-deficient mice, indicating a more profound defect of host defense in the former animals. In contrast, a high bacterial dose induced high level bacteremia in both mutant and wild-type mice. Under these conditions, however, TLR2 or MyD88 deficiency significantly protected mice from lethality, concomitantly with decreased circulating levels of TNF- α and IL-6. Administration of anti-TNF- α Abs to wild-type mice could mimic the effects of TLR2 or MyD88 deficiency and was detrimental in the low dose model, but protective in the high dose model. In conclusion, these data highlight a dual role of TLR2 and MyD88 in the host defense against GBS sepsis and strongly suggest TNF- α as the molecular mediator of bacterial clearance and septic shock. *The Journal of Immunology*, 2004, 172: 6324–6329.

Group B streptococci (GBS)³ are a frequent cause of systemic infections in neonates and adults with underlying chronic conditions (1, 2). The incidence of lethality and permanent neurological damage is high despite the availability of modern therapy. Several observations suggest that an exaggerated production of proinflammatory cytokines is responsible for many of the manifestations of GBS sepsis. In neonates with early onset GBS sepsis, plasma levels and gene expression of proinflammatory cytokines are extremely elevated (3, 4). Experimental rodent models of GBS disease indicate that TNF- α has a crucial role in mediating lethality, as evidenced by the protective effects of prophylactic administration of anti-TNF- α Abs (5, 6). Finally, GBS most potently activate phagocytes, as evidenced by maximal cytokine levels induced in vitro using human monocytes or mouse macrophages (7, 8).

Toll-like receptors (TLRs) belong to a family of evolutionarily conserved type I transmembrane proteins capable of detecting the presence of microbial products or damaged host tissues (9). A leucine-rich domain, which is presumably responsible for ligand recognition, is present in the extracellular portion of TLRs,

whereas the cytoplasmic portion contains a region that is highly homologous to the intracellular signaling domain of the IL-1R (the so-called TIR domain). Activation of TLRs initiates a cascade of intracellular events resulting in cytokine production and the expression of costimulatory molecules (10). There is strong evidence that TLRs play a crucial role in innate immunity responses, including inflammation, activation of antimicrobial mechanisms, and initiation of adaptive immune responses (9–11).

Different TLRs are engaged by different microbial stimuli (9–11). The LPS component of the Gram-negative cell wall activates TLR4 in conjunction with the secreted molecule MD2. Bacterial flagellin and bacterial DNA activate TLR5 and TLR9, respectively. TLR2 can recognize, in conjunction with TLR6 or TLR1, a wide array of microbial products, including peptidoglycan, lipoproteins, and glycolipids from bacteria, as well as yeast cell walls and mycobacterial products (8–11). This latter observation suggested that TLRs have the ability to establish a combinatorial repertoire to discriminate among the large number of different microbial products, although to date such a combinatorial repertoire has only been found for TLR2 and its partners.

The signaling pathways activated by TLRs have been well characterized and include at least five different TIR domain-containing adapter molecules, protein kinases, and transcription factors. Myeloid differentiation factor 88 (MyD88) is the best studied of the adaptor molecules and has an important role in transducing activation signals from TLRs and the IL-1R (12, 13). MyD88-deficient mice are more susceptible to infection by different pathogens (14–18), indicating a crucial role of MyD88 in antimicrobial defenses. Surprisingly, MyD88-deficient mice have also been reported to be more resistant to the effects of septic polymicrobial peritonitis, implying a pathophysiological role of MyD88 in sepsis (19). These apparently conflicting observations in the MyD88 knockouts raise the possibility that the responses to septic insults mediated by MyD88 depend upon the precise conditions of infection and may differ substantially based on inoculum, the location of the infection, and other variables that are, as yet, poorly understood.

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³ Abbreviations used in this paper: GBS, group B streptococci; MyD88, myeloid differentiation factor 88; TIR domain, intracellular domain of the TLR/IL-1R; TLR, Toll-like receptor; WT, wild type.

Comparatively little is known about the mechanisms of cellular activation in GBS-induced cytokine production relative to the many observations that have been made with other microbial stimuli, such as LPS or peptidoglycan. Studies using peritoneal macrophages from MyD88- or TLR2-deficient mice indicate that TLR2 is involved in cytokine responses to extracellular GBS products, but not to whole bacteria, whereas MyD88 plays an essential role in responses to both types of stimuli (20). In view of the potentially important functions of TLR2 and MyD88 in anti-GBS defenses, we assessed the roles of these molecules in GBS-infected MyD88- or TLR2-deficient mice. The results provide compelling evidence of the dual role of these signaling molecules as important components of the host defense system during initial infection and as mediators of lethality in the presence of overwhelming sepsis.

Materials and Methods

Mice

MyD88^{-/-} and TLR2^{-/-} mice were engineered as described on a 129 SV-C57BL/6 background (21–23) and were backcrossed five times with C57BL/6 mice. Control wild-type (WT) C57BL/6 mice were purchased from Charles River Italia (Calco, Italy).

Experimental models of GBS disease

Both neonatal and adult models of GBS disease were studied. In the neonatal model, 24- to 48-h-old MyD88^{-/-}, TLR2^{-/-}, or control C57BL/6 pups were injected s.c. with the highly virulent type III strain COH1, as previously described (24, 25). Bacteria were grown to the mid-log phase in Todd-Hewitt broth (Oxoid, Milan, Italy) and were diluted to the appropriate concentration in PBS (0.01 M phosphate and 0.15 M NaCl, pH 7.2) before inoculation of animals. In each experiment the number of injected bacteria was carefully determined by colony counts on blood agar (Oxoid).

In the adult model, 6-wk-old mice of either sex were injected i.p. with GBS type V strain 2603 V/R (26). Again, the actual number of injected bacteria was determined by colony counts. Mice were observed daily for 8 days after inoculation. Deaths were never observed after 5 days. In addition, some of the mice were sacrificed at the indicated intervals to measure bacterial burden and plasma cytokine levels.

The studies presented in this study were approved by the relevant national and local ethical review boards.

Measurement of bacterial burden

Mice were sacrificed at 24 or 48 h after challenge, and colony counts were measured in the blood, spleen, and kidney. Blood (10 μ l) was diluted 1/20 in PBS, whereas spleens and kidneys were homogenized in PBS. Serial dilutions were prepared in duplicate and plated on blood agar. Bacterial CFU were assessed after overnight growth at 37°C.

Cytokine determinations

TNF- α and IL-6 concentrations were determined in duplicate in plasma samples using commercial ELISA kits (mouse TNF- α module set (Bender MedSystems, Vienna, Austria) and murine IL-6 reagent set (Euroclone, Wetherby, U.K.)). The lower detection limit of both these assays was 16 pg/ml. Neonatal plasma was diluted 1/10 before the assay, and thus the detection limits were 10 times higher.

Anti-TNF- α treatment

To study the effects of TNF- α blockade, neonatal mice were pretreated, at 6 h before GBS challenge, with 20 μ g of polyclonal anti-TNF- α or normal goat IgG, both purchased from R&D Systems (Minneapolis, MN). Anti-TNF- α or normal IgG was diluted in PBS, and 25 μ l was injected s.c. It was found in preliminary experiments that 4 μ g/pup was the minimal anti-TNF- α dose resulting in complete abrogation of TNF- α bioactivity in plasma samples collected at 24 and 48 h after challenge with 60 CFU of the strain COH-1 strain. TNF- α bioactivity was measured by a cytotoxicity assay using the WEHI 164 clone 13 cell line, as previously described (27).

Data expression and statistical significance

Survival data were analyzed with Kaplan-Meier survival plots, followed by the Wilcoxon test. Cytokine levels and log CFU were expressed as the mean \pm SD of five determinations, each conducted on a different animal. Differences in plasma cytokine levels and organ CFU were assessed by

one-way ANOVA and Student-Newman-Keuls test. With both tests, differences were considered significant at $p < 0.05$.

Results

Increased susceptibility of MyD88- and TLR2-deficient mice to low dose GBS infection

WT, TLR2^{-/-}, or MyD88^{-/-} neonatal mice were injected s.c. with a low dose (3 CFU) of GBS inoculum, and lethality was measured over time (Fig. 1). Under these conditions, all WT mice survived, whereas 20% of the TLR2-null and 30% of the MyD88-null mice died ($p < 0.05$ for both groups of mice vs WT). The differences in lethality between MyD88- and TLR2-deficient pups were not statistically significant. In addition to the assessment of mortality, bacterial burden and plasma cytokine levels were measured at 24 and 48 h after infection with the same low dose inoculum (3 CFU). No bacteria were detected in the blood, kidney, or spleen of any of the WT mice, indicating that these animals were able to prevent the systemic spread of GBS from the site of inoculation. In contrast, bacteria were detected in the blood, kidney, and spleen of each of the genetically deficient mice after challenge (Fig. 1). Moreover, significantly increased bacterial titers were observed in the blood and organs of MyD88^{-/-} pups, relative to TLR2^{-/-} animals, indicating a more profound deficiency of anti-streptococcal defenses in the MyD88^{-/-} animals (Fig. 1).

None of the WT animals had detectable circulating cytokine levels, probably reflecting the absence of bacteremia (Fig. 1). Elevated TNF- α and IL-6 levels were found in the blood of 100 and 60%, respectively, of TLR2^{-/-} and MyD88^{-/-} mice. Increased mean cytokine levels were found in TLR2-deficient pups, relative

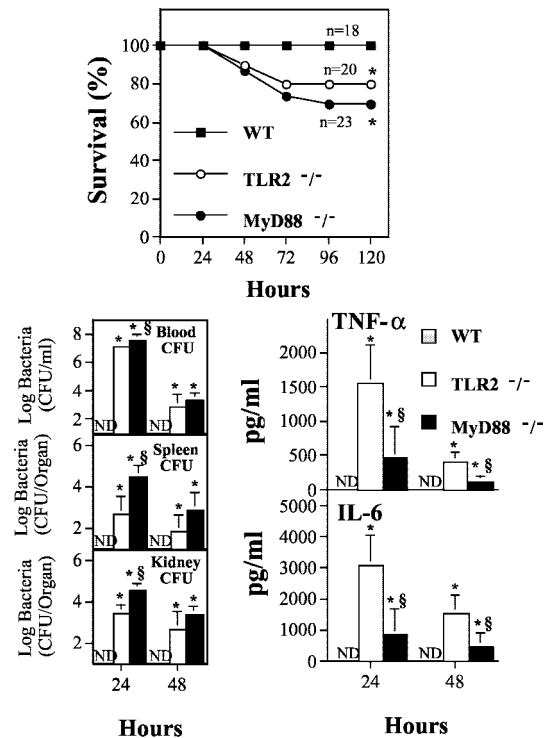
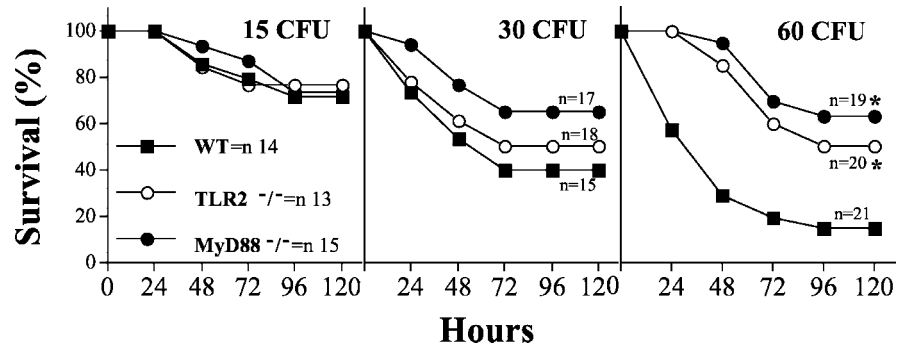


FIGURE 1. Upper panel, Survival of WT, TLR2-deficient, and MyD88-deficient neonatal mice after challenge with a low dose (3 CFU) GBS inoculum. Lower panels, Bacterial numbers (left panel) and cytokine levels (right panel) in WT, TLR2-deficient, and MyD88-deficient neonatal mice after challenge with a low dose (3 CFU) GBS inoculum. Cytokine levels and log CFU were expressed as the mean \pm SD of five determinations, each conducted on a different animal. * and $\$$, $p < 0.05$ vs WT and TLR2-deficient mice, respectively. ND, nondetectable.

FIGURE 2. Survival of WT, TLR2-deficient, and MyD88-deficient neonatal mice after challenge with different GBS inocula (15, 30, and 60 CFU, from left to right). *, $p < 0.05$ vs WT mice.



to MyD88-deficient ones, despite the fact that the latter mice had higher bacterial counts (Fig. 1). This indicated a more profound defect in cytokine responses to GBS in MyD88-null, relative to TLR2-null, mice.

Increased resistance of MyD88- and TLR2-deficient mice to overwhelming GBS sepsis

It was reported recently that MyD88 deficiency resulted in improved survival in a mouse model of polymicrobial sepsis (19). These studies were conducted under conditions that resulted in high (80%) lethality in WT mice. To determine whether the effects of gene expression might be qualitatively different under varying conditions of control lethality, WT, TLR2^{-/-}, and MyD88^{-/-} pups were infected with increasing doses of GBS (15, 30, and 60 CFU). These inocula resulted in ~30, 60, and 90% lethality, respectively, in WT controls (Fig. 2). No statistically significant differences were detected in the final survival of WT, TLR2^{-/-}, and MyD88^{-/-} mice challenged with the 30 and 60% lethal doses. In contrast, at the highest dose of GBS tested, 86% of the WT mice died. Significant protection was observed in both TLR2^{-/-} and MyD88^{-/-} mice (Fig. 2). Thus, TLR2^{-/-} or MyD88^{-/-} deficiency protected mice from lethal sepsis after a high dose challenge.

In view of the phenotype of the knockout mice injected with GBS, bacterial counts and plasma cytokine levels were measured in animals infected with the “high dose” challenge (60 CFU). Fig. 3 shows that elevated bacterial counts were found in all animals, with no differences in the bacterial burden measured in blood,

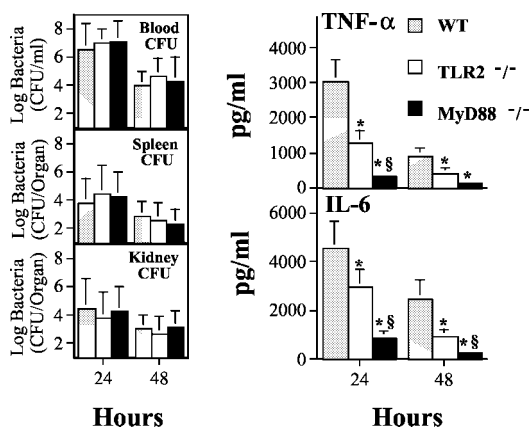


FIGURE 3. Bacterial numbers (left panel) and cytokine levels (right panel) in WT, TLR2-deficient, and MyD88-deficient neonatal mice after challenge with a high dose (60 CFU) GBS inoculum. Cytokine levels and log CFU were expressed as the mean \pm SD of five determinations, each conducted on a different animal. * and §, $p < 0.05$ vs WT and TLR2-deficient mice, respectively.

spleen, and kidney between pups of different genotypes. However, plasma TNF- α and IL-6 levels were significantly reduced in both strains of knockout mice relative to WT ones. In addition, cytokine values were decreased in MyD88-null pups, relative to the TLR2-null animals, confirming that MyD88 deficiency, which affects multiple TLRs, results in a more profound defect in cytokine responses than the simple loss of TLR2. This is in agreement with previous *in vitro* studies indicating the involvement of multiple TLRs in cytokine responses to GBS (20).

Roles of MyD88 and TLR2 in adult GBS infection

Host defenses are profoundly altered in the neonatal period, as is the susceptibility to shock induced by different bacterial products (27). Therefore, it could not be excluded that the variable susceptibility to GBS disease observed in the mutant pups was related to their age. Moreover, serious GBS infections are frequent not only in neonates, but also in adults with underlying chronic diseases (1, 2). Thus, we tested the innate resistance to GBS of MyD88- and TLR2-defective adult mice.

One obvious and important difference between neonatal and adult mice is that inocula of $\leq 10^5$ CFU have no apparent detrimental effect in WT adult mice (G. Teti, unpublished observations). This correlated with the relative infrequency of GBS infections in adults without underlying conditions, such as pregnancy or chronic disease (1, 2).

To test the role of TLR2/MyD88 in adult GBS infection, 6-wk-old TLR2^{-/-}, MyD88^{-/-}, or WT mice were inoculated *i.p.* with three different doses (5×10^5 , 5×10^6 , and 5×10^7 CFU) of type V GBS, a serotype that is frequently associated with adult human GBS disease. The lower dose resulted in no lethality in either WT and genetically defective mice (not shown). The intermediate dose (5×10^6 CFU) resulted in no lethality in WT mice, but killed 25 and 33% of TLR2^{-/-} and MyD88^{-/-} mice, respectively (Fig. 4). When mice were infected with the high dose inoculum (5×10^7 CFU), which resulted in 90% lethality in control mice, MyD88- or TLR2-deficient mice were significantly protected against lethality (Fig. 4), relative to WT mice, in association with decreased plasma TNF- α levels (Fig. 4, middle panel).

In additional experiments, blood colony counts were determined in mice inoculated with the three different GBS doses (5×10^5 , 5×10^6 , or 5×10^7 CFU). None of the WT or genetically defective mice developed bacteremia after inoculation with the lowest dose (5×10^5 CFU; not shown). However, after inoculation with the intermediate dose (5×10^6 CFU), higher blood colony counts were observed in TLR2- or MyD88-deficient mice, relative to WT ones (Fig. 4, lower left panel). Moreover, under these conditions, MyD88-deficient mice showed significantly higher colony counts than TLR2-deficient ones. The different groups of mice showed similar high level bacteremia when inoculated with the highest dose (Fig. 4, lower right panel). Collectively, these data indicated

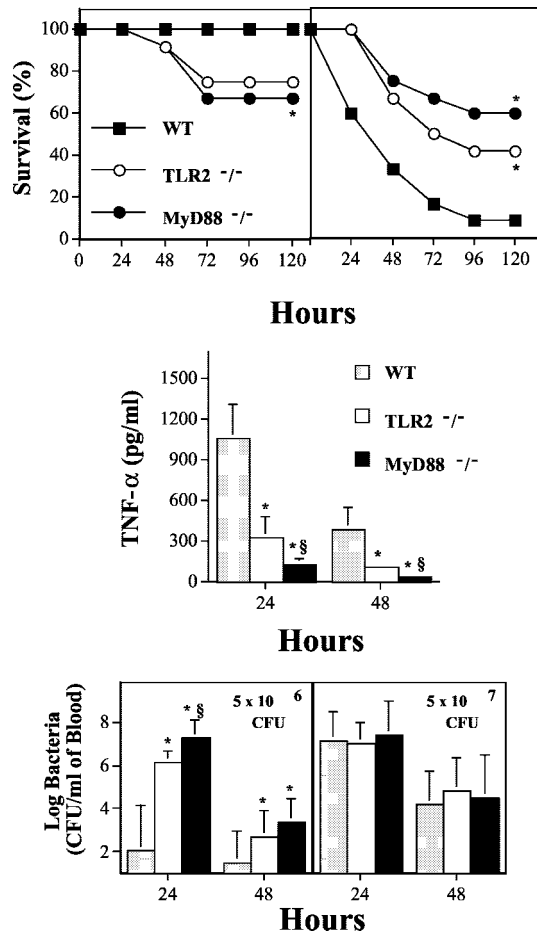


FIGURE 4. Survival of WT, TLR2-deficient, and MyD88-deficient adult mice after challenge with an intermediate (5×10^6 CFU; upper left panel) or a high (5×10^7 CFU; upper right panel) dose GBS inoculum. Plasma TNF- α levels in WT, TLR2-deficient, and MyD88-deficient adult mice inoculated with a high dose GBS inoculum (5×10^7 CFU; middle panel). Blood colony counts in WT, TLR2-deficient, and MyD88-deficient adult mice after challenge with an intermediate (5×10^6 CFU; lower left panel) or a high (5×10^7 CFU; lower right panel) dose GBS inoculum. Cytokine levels and log CFU were expressed as the mean \pm SD of five determinations, each conducted on a different animal. * and §, $p < 0.05$ vs WT and TLR2-deficient mice, respectively.

that MyD88 or TLR2 deficiency had similar effects in neonatal and adult mice in terms of their susceptibility to GBS infection.

Effects of TNF- α blockade on GBS-induced lethality

Previous studies have indicated a major role of TNF- α in mediating lethality of animals rendered septic with GBS. This was evidenced by the protective effects of anti-TNF- α (5, 6), but not anti-IL-6 (25), Abs in neonatal rodents infected with high GBS inocula. However, the effects of TNF- α blockade were not previously studied using a low dose GBS challenge. As, in the present study, MyD88 or TLR2 deficiency resulted in decreased TNF- α responses, it was of interest to ascertain whether the phenotype of MyD88- or TLR2-deficient mice could be mimicked by administration of anti-TNF- α Abs. To this end, neonatal WT C57BL/6 mice were inoculated with anti-mouse TNF- α goat IgG 6 h before challenge with different GBS doses. Control animals received normal goat IgG. In strong support of our initial hypothesis, TNF- α blockade resulted in increased (26%) lethality in mice inoculated with the low GBS inoculum, whereas all the control mice survived (Fig. 5, upper left panel). In contrast, the same anti-TNF- α treat-

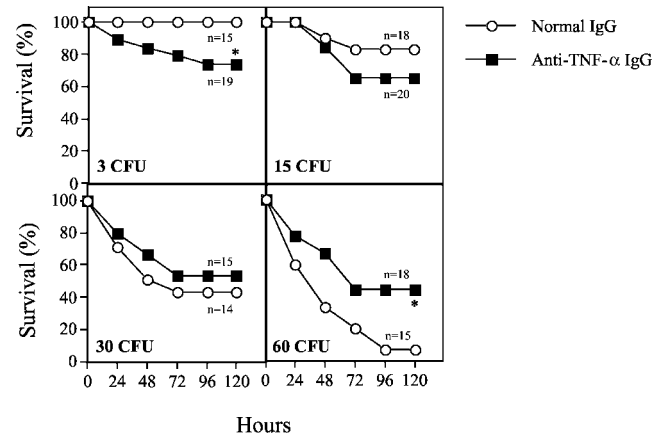


FIGURE 5. Effects of TNF- α blockade on survival of neonatal mice challenged with different GBS inocula. Pups were injected s.c. 6 h before GBS challenge with 20 μ g of polyclonal anti-TNF- α or normal goat IgG. *, $p < 0.05$ vs normal IgG-treated neonatal mice.

ment had protective effects in mice inoculated with the highest GBS doses (Fig. 5, lower right panel). No significant effects on lethality were noted when mice were inoculated with intermediate doses (15 or 30 CFU; Fig. 5). In additional experiments, we determined the number of CFU in the blood and kidneys of mice pretreated with anti-TNF- α or normal IgG before challenge. Fig. 6 shows that significantly higher colony counts were found in anti-TNF- α -treated pups relative to control mice at 24 h after challenge with 3 or 15 CFU. Using higher GBS inocula, both groups of mice showed similar, high level bacteremia. Thus, the effects of TNF- α blockade on both lethality and colony counts were very much reminiscent of those previously observed in MyD88- or TLR2-null mice (compare Figs. 5 and 6 with Figs. 1 and 3). This observation suggested that the phenotype of MyD88- or TLR2-null mice is secondary to blunted TNF- α responses during GBS infection.

Discussion

At least one of 10 infants acquires GBS by vertical or horizontal transmission during the first month of life (1, 2, 28). The vast majority of these individuals have mucosal colonization without apparent illness. Only 1% of the colonized neonates develop systemic GBS infection, indicating the presence of effective host defenses in most individuals. The elimination of invading GBS can be inferred to rest exclusively on the innate immune system, because protective levels of opsonizing Abs are rarely present in neonates (28). This same defense system may, however, mediate a lethal septic shock syndrome in response to overwhelming GBS challenge (3–5).

Previous *in vitro* studies indicated that both TLR2 and MyD88 are involved in GBS-induced cell activation and cytokine production (20). These studies implicated additional members of the TLR family, including TLR6 as well as at least one additional, and an as of yet unidentified, TLR. To determine the relevance of the *in vitro* observations to the pathogenesis group B streptococcal disease, the present study examined the *in vivo* requirements for TLR2 and MyD88 in host resistance to GBS. To this end, we have systematically explored the effects of a wide range of bacterial doses on differential lethality of MyD88- or TLR2-deficient mice.

The model using the lowest dose was designed to mimic the initial exposure of subepithelial tissues to GBS in newborns. Under these conditions, TLR2 or MyD88 deficiency markedly decreased the host ability to control systemic bacterial spread from the site of infection, suggesting that the Toll receptor system has a central role in

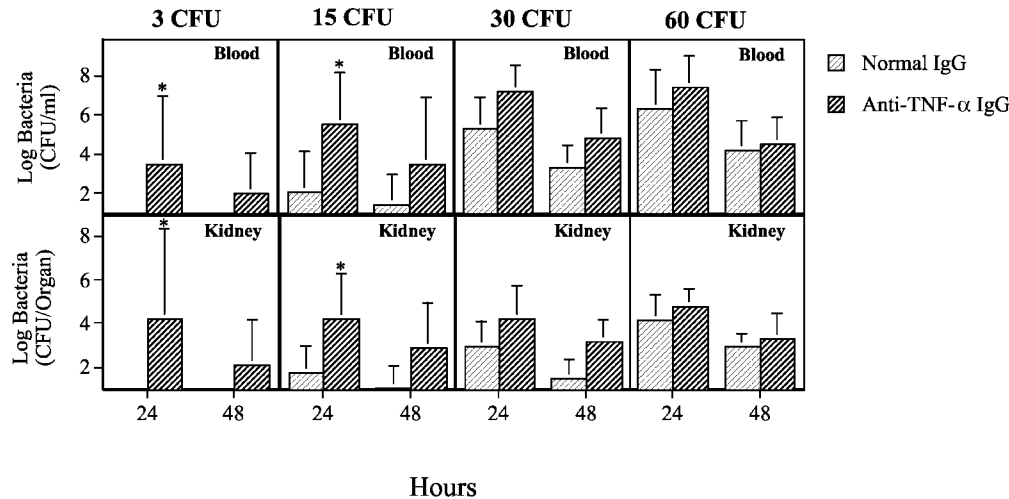


FIGURE 6. Effects of TNF- α blockade on bacterial burden in neonatal mice challenged with different GBS inocula. Pups were pretreated 6 h before GBS challenge with 20 μ g of polyclonal anti-TNF- α or normal goat IgG. Cytokine levels and log CFU were expressed as the mean \pm SD of five determinations, each conducted on a different animal. *, $p < 0.05$ vs normal IgG-treated neonatal mice.

the surveillance against GBS invasion. The failure of the innate immune system to detect traces of microorganisms before systemic invasion has been shown to be detrimental in various mouse models. For example, LPS hyporesponder mice, including TLR4, CD14, and LPS binding protein mutants, could not control infection by a very small inoculum of *Salmonella typhimurium* (14, 29–31).

In the present study MyD88 deficiency was associated with a more profound inability to either control infection or produce cytokine responses relative to TLR2 deficiency. These data suggest that in addition to TLR2, other receptors using the MyD88 pathway are involved in GBS-induced activation of the innate immune system. This is in agreement with *in vitro* observations that whole bacterial cells use an as yet undefined MyD88-dependent, but TLR2-independent, pathway, whereas TLR2 is involved in cell activation by extracellular GBS products (20). Thus, our data are compatible with the idea that both bacterial cells and secreted products are important *in vivo* activators of the immune system during infection. It should be noted, however, that MyD88-deficient mice are unable to respond not only to TLR activation, but also to IL-1 or IL-18 (4, 5). Because these cytokines are important mediators in host defense, it cannot be excluded that the inability to respond to IL-1 and IL-18 also contributed to the phenotype of MyD88-deficient mice.

Paradoxically, in the present study TLR2 or MyD88 deficiency protected mice from lethality against a high dose challenge, which, unlike a low dose challenge, was associated with similar levels of bacteremia in all three groups of mice. Under these conditions, circulating levels of TNF- α and IL-6 were markedly reduced in the genetically deficient mice. These data indicate that in the presence of overwhelming sepsis, TLR2 or MyD88 contributes to an excessive and lethal cytokine storm, and deficiency of those molecules improves outcome.

Our data highlight the importance of using a full range of infecting doses in experimental sepsis studies. The effects of MyD88 or TLR2 mutations on lethality would have been missed in this study using only one intermediate (30–60% lethal) dose. Higher doses would have detected only the protective effects of these mutations on lethal sepsis, but not the associated decreased ability to control local infection. Our findings are in strong agreement with those of a recent study showing that a variety of anti-inflammatory agents could improve and worsen the survival of animals inoculated with, respectively, high and low bacterial inocula (32). In that

study a strong relationship was found between risk of death and treatment effects of anti-inflammatory agents regardless of the type of agent or the route and type of challenge (32). Notably, a similar effect was shown in a metaregression analysis of preclinical and clinical trials. Anti-inflammatory agents were efficacious only in septic patients with a higher risk of death, but were harmful in those with a low risk (32, 33).

Apparently, innate immunity differentially affects sepsis outcome depending on the severity of the underlying infectious process and the associated risk of death. In situations in which the overwhelming majority of subjects are likely to survive (e.g., in neonates colonized with GBS or affected by mild GBS disease), invasion is kept in check by a vigilant and highly reactive innate immune system. Under these conditions, immune defects or immunosuppressive treatments can prevent a well-regulated response and weaken vital host defenses. Conversely, once a substantial bacterial invasion has been established, the presence of an immune defect or anti-inflammatory therapies may inhibit a lethal cytokine storm and improve survival. These two clinical situations can be mimicked experimentally using, respectively, low and high control lethality rates (32). With intermediate control lethality rates, i.e., with a mixture of subjects destined either to die or to survive, the beneficial effects of avoiding hyperinflammation in the former may be obscured by damage related to disruption of host defenses in the latter. Accordingly, in the present study no differences were found between genetically deficient and WT animals in susceptibility to GBS disease using intermediate control lethality rates.

In conclusion, our results indicate that both TLR2 and MyD88 are essential components of anti-GBS defenses during initial, localized infection, but can become mediators of lethality in the presence of overwhelming sepsis. These data experimentally support the idea that innate immunity differentially affects sepsis outcome according to the severity of underlying infectious process and the associated risk of death.

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