

MyD88-Dependent Signaling Affects the Development of Meningococcal Sepsis by Nonlipooligosaccharide Ligands

Laura Plant,^{1,2*} Hong Wan,¹ and Ann-Beth Jonsson¹

Department of Medical Biochemistry and Microbiology, Biomedical Centrum, Uppsala University, 751 23 Uppsala, Sweden,¹ and Smittskyddsinstitutet, Swedish Institute for Infectious Disease Control, Karolinska Institutet, Nobelsväg 18, 171 77 Solna, Sweden²

Received 25 January 2006/Returned for modification 9 March 2006/Accepted 24 March 2006

The Toll-like receptors (TLRs) and the adaptor myeloid differentiation factor 88 (MyD88) are important in the innate immune defenses of the host to microbial infections. Meningococcal ligands signaling via TLRs control inflammatory responses, and stimulation can result in fulminant meningococcal sepsis. In this study, we show that the responses to nonlipooligosaccharide (non-LOS) ligands of meningococci are MyD88 dependent. An isogenic LOS-deficient mutant of the serogroup C meningococcal strain FAM20 caused fatal disease in wild type C57BL/6 mice that was not observed in MyD88^{-/-} mice. Fatality correlated with high proinflammatory cytokine and C5a levels in serum, high neutrophil numbers in blood, and increased bacteremia at 24 h postinfection in the wild-type mice. Infection with the parent strain FAM20 resulted in fatality in 100% of the wild-type mice and 50% of the MyD88^{-/-} mice. We conclude that both LOS and another neisserial ligand cause meningococcal sepsis in an in vivo mouse model and confirm that meningococcal LOS can act via both the MyD88-dependent and -independent pathways, while the non-LOS meningococcal ligand(s) acts only via the MyD88-dependent pathway.

Neisseria meningitidis is a commensal inhabitant of the upper respiratory tract of between 5 and 15% of humans (8, 30). Nasopharyngeal carriage of meningococci is normally entirely asymptomatic, and meningococemia and sepsis occur in only a small percentage of colonized people. The development of invasive disease is thought to depend on host factors, and despite improvement in treatment, *N. meningitidis* is still an important cause of morbidity and mortality around the world.

The Toll-like receptors (TLRs) are one of the most important families of signaling receptors in the innate immune defenses of the host to microbial infections (41). TLRs control a range of events, including the induction of proinflammatory cytokines, chemokines, costimulatory molecules, and adhesion molecules. The induction of these factors can be beneficial, by stimulating cellular responses to the infection, as well as potentially harmful, since uncontrolled proinflammatory responses can lead to sepsis and fatality. At least 13 members of the TLR family have been identified in mammals. These recognize pathogen-associated molecular patterns (PAMPs), which are conserved molecules shared among a broad range of pathogens. After recognition of ligands, the TLRs can activate a common signaling pathway that leads to activation of the NF- κ B transcription factor and mitogen-activated protein kinase signaling molecules.

Myeloid differentiation factor 88 (MyD88) is an essential adaptor molecule for responses to a broad range of microorganisms or their components that are recognized by TLR2, TLR4, TLR5, TLR7, or TLR9. Some TLRs (including TLR3 and TLR4) signal via MyD88-independent pathways (42). Three other adaptor proteins associated with the TLRs have

also been identified, and these include TRIF (Toll receptor-associated adaptor of interferon), MAL/TIRAP (MyD88-adaptor-like/ Toll/interleukin 1 [IL-1] receptor homologous region [TIR]-associated protein), and TRAM (Toll-receptor-associated molecule). All transduce signals from the TIR domains, activating protein kinases and transcription factors that cause inflammatory effects.

Neisseria meningitidis expresses a variety of PAMPs recognized by the TLRs. The highly abundant outer membrane (OM) protein PorB activates dendritic cells in a TLR2- and MyD88-dependent manner (24, 35). Similarly, the closely related species *Neisseria gonorrhoeae* expresses lipopeptides recognized by TLR2 (13). Meningococcal lipooligosaccharide (LOS) and OM blebs containing LOS have been reported to stimulate CD14/TLR4 (25, 46) via both MyD88-dependent and MyD88-independent pathways (47).

LOS has long been presumed to be the major inflammatory mediator of fulminant meningococcal sepsis and meningitis, with disease severity correlating with circulating concentrations of LOS and proinflammatory cytokines (4). Meningococcal LOS lacks the repeating O antigens of enteric LPS, but maintains a conserved inner core composed of heptose and 3-deoxy-D-manno-2-octulosonic acid (KDO) bound to lipid A, to which variable α - and β -chain saccharides are attached (18). Lipid A is the active moiety through its ability to upregulate the inflammatory response (34). Development of meningococcal LOS knockout mutants has allowed the study of the contribution of non-LOS molecules to the host response (39, 40, 44). The contributions of non-LOS ligands of meningococci to cellular responses have been shown in a number of studies to have effects on cytokine induction (10, 16, 32, 40, 43, 46), endothelial cell adhesion molecule expression (9), and expression of stress response genes (3). It has been shown that an LOS-deficient strain of *N. meningitidis* activates macrophages through TLR2 (17, 32); however, another study recently

* Corresponding author. Mailing address: Smittskyddsinstitutet, Swedish Institute for Infectious Disease Control, Karolinska Institutet, Nobelsväg 18, 171 77 Solna, Sweden. Phone: 46 8457 24 32. Fax: 46 830 25 66. E-mail: Laura.Plant@smi.ki.se.

showed that the recognition of the LOS-deficient OM of *N. meningitidis* is not associated with the expression of TLR2 on human meningeal cells (16). The involvement of other pathogen response receptors in meningococcal infection, including the class A macrophage scavenger receptor SR-A (31) and other unidentified receptors, has also been suggested (16).

To investigate the role of MyD88 in the pathway of stimulation in response to non-LOS factors *in vivo*, we infected MyD88^{-/-} mice with the serogroup C meningococcal strain FAM20 and an LOS-deficient isogenic *lpxA* strain (1). Our data show that MyD88 is crucial in the development of meningococcal sepsis associated with the unidentified non-LOS neiserial ligand(s).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The serogroup C meningococcal strain *N. meningitidis* FAM20 (12, 33) and the LOS-deficient FAM20 (*lpxA*) mutant (1) have previously been described. The *lpxA* mutant has a disrupted *lpxA* gene that encodes an enzyme responsible for the first step of the lipid A biosynthesis. Both strains were grown on GC agar containing Kellogg's supplement (20) at 37°C in a 5% CO₂ atmosphere for 18 to 20 h, and the GC agar used for growth of the LOS mutant was supplemented with 50 µg of kanamycin/ml. Meningococcal strains were suspended in prewarmed GC liquid and immediately used for mouse inoculation.

Media and buffers. The cell culture medium was Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% inactivated fetal bovine serum (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Gibco), and 1× nonessential amino acids (Gibco). Medium was supplemented with the antibiotics penicillin (100 U/ml) and streptomycin (100 U/ml) where indicated. GC liquid was composed of 1.5% (wt/vol) protease peptone no. 3 (Becton Dickinson), 3 mM soluble starch (Sigma), 23 mM K₂HPO₄ (Merck), 7 mM KH₂PO₄ (Merck), and 50 mM NaCl (Merck).

Mouse strains. C57BL/6 mice (B6; wild type) were obtained from Taconic (M&B), Ry, Denmark. MyD88^{-/-} mice (backcrossed 6 generations on the B6 background) were kept under pathogen-free conditions. All mice were bred and housed at the animal facility at the Rudbeck's Laboratory, Uppsala, Sweden. Mice were 5 to 8 weeks old when challenged with bacteria. All animal procedures were performed in accordance with the institutional guidelines of Uppsala University under an approved protocol (C264/4).

Animal model. Five- to 8-week-old mice were randomly distributed in groups and injected intraperitoneally (i.p.) with 100 µl of a bacterial suspension containing 1 × 10⁸ or 5 × 10⁸ FAM20 cells and the *lpxA* mutant strain. Equivalent amounts of bacteria were determined by optical density at 600 nm. The optical density measurement was used to control the dose since the *lpxA* mutant strain exhibits clumping and viable counts of this organism are lower at an equivalent optical density. The number of viable bacteria in the challenge dose was confirmed by viable count. Blood samples were obtained from the tail at different time points after bacterial challenge. The skin was disinfected with 70% ethanol, and 5 µl of blood was collected from the tail. Blood was diluted in GC liquid, serial dilutions were plated on GC agar plates and incubated overnight at 37°C in a 5% CO₂ atmosphere, and CFU were enumerated.

Serum collection. Mice that had been infected with 1 × 10⁸ wild-type FAM20 cells or equivalent amounts of biological material of the *lpxA* mutant strain were anesthetized by light inhalation of isoflurane (Forene; Abbott), and blood was collected by retro-orbital bleed at various times postinfection. Blood was allowed to clot at 4°C, and serum was collected after centrifugation at 10,000 × g for 10 min. Serum was stored at -70°C until used.

Determination of cytokine and chemokine levels in serum by ELISA. Concentrations of murine interleukin 6 (IL-6), tumor necrosis factor (TNF), IL-10, gamma interferon (Diacalone), KC, IFN-γ-inducible protein 10 (IP-10), and IFN-β (R&D Systems) were measured in serum of *N. meningitidis*-challenged mice (*n* ≥ 6) at different time points postinjection by using sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's recommendations.

Analysis of C5a levels in serum by ELISA. For detection of C5a in mouse serum, a rat anti-mouse C5a monoclonal antibody, I52-1486 (1 µg/ml), and biotinylated rat anti-mouse C5a monoclonal antibody I52-278 (0.5 µg/ml) from BD Biosciences were used, respectively, as ELISA capture and detection antibodies. Serum from infected mouse groups (*n* > 6) was diluted 1:10 in 1% bovine

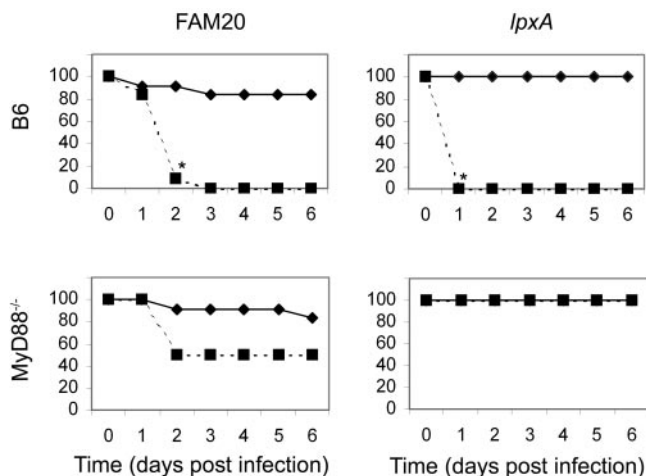


FIG. 1. MyD88^{-/-} mice are resistant to intraperitoneal challenge with the LOS-deficient meningococcal *lpxA* mutant. Shown is the survival of B6 and MyD88^{-/-} mice following infection with 1 × 10⁸ CFU/mouse (solid lines; *n* = 12) and 5 × 10⁸ CFU/mouse (dashed lines; *n* = 12) of the wild-type bacterial strain *N. meningitidis* FAM20 or equivalent amounts of the LOS-deficient *lpxA* mutant. Survival was analyzed by Fisher's exact test. Significant differences between the low- and high-dose survival are indicated by an asterisk at the time point at which the differences became significant (*P* < 0.05).

serum albumin in phosphate-buffered saline and incubated for 2 h at room temperature. Serum from the uninfected B6 mice was arbitrarily assigned a concentration of 40 U/ml C5a.

Blood smears. Blood was obtained from the tail veins of mice at various time points postinfection of meningococci as described above, and smears were made. Slides were fixed with methanol and stained with Wright's stain according to manufacturer's recommendations (Sigma). A differential white blood cell count was performed by light microscopy.

Whole-blood bactericidal assay. Whole blood was taken from the orbital vein of B6 and MyD88^{-/-} mice (*n* = 3), pooled, and mixed 1:5 with sodium citrate (0.1 M). Bacterial suspensions containing 5 × 10⁵ CFU of *N. meningitidis* FAM20 or the *lpxA* mutant were mixed 1:1 with blood in a final volume of 200 µl. The mixtures were incubated in 5% CO₂ and 37°C for 1 h and 4 h, and surviving bacteria were plated on GC agar. The surviving viable bacteria in blood were determined after incubating plates overnight in 5% CO₂ and 37°C.

Phagocytosis of meningococci by peritoneal macrophages. Peritoneal cells were collected from uninfected B6 and MyD88^{-/-} mice (*n* = 3). Briefly, animals were sacrificed by cervical dislocation, and 5 ml of cold sterile phosphate-buffered saline was injected intraperitoneally. Recovered cells were pelleted by centrifugation at 400 × g for 10 min at 4°C, washed, and cultured in cell culture medium containing antibiotics overnight in 5% CO₂ and 37°C. For the infection of cells, the antibiotics were removed by washing cells with cell culture media without antibiotics and bacteria were added in fresh cell culture media at a multiplicity of infection of 100. After 60 min of incubation, extracellular bacteria were killed with 250 µg/ml gentamicin for 1 h at 37°C. The cells were lysed with 200 µl of 1% saponin (Quillaja bark; Sigma) for 10 min, and 800 µl of prewarmed GC liquid was added to the wells. Bacteria were enumerated by plating on GC agar.

Statistical analysis. Data were evaluated using Microsoft Excel. A two-tailed Student's *t* test was used to assess significance in the blood survival assay, macrophage phagocytosis assay, and cytokine assays. Lethality experiments were assessed using a Fisher's exact test, and bacteremia and neutrophil numbers were monitored with a nonparametric Mann-Whitney test. Significance was accepted at *P* < 0.05. All results were obtained in at least two independent experiments.

RESULTS

MyD88^{-/-} mice show resistance to meningococcal sepsis. To assess the role of MyD88 in the development of meningococcal sepsis, the survival of wild-type C57BL/6 (B6) mice and

FAM20

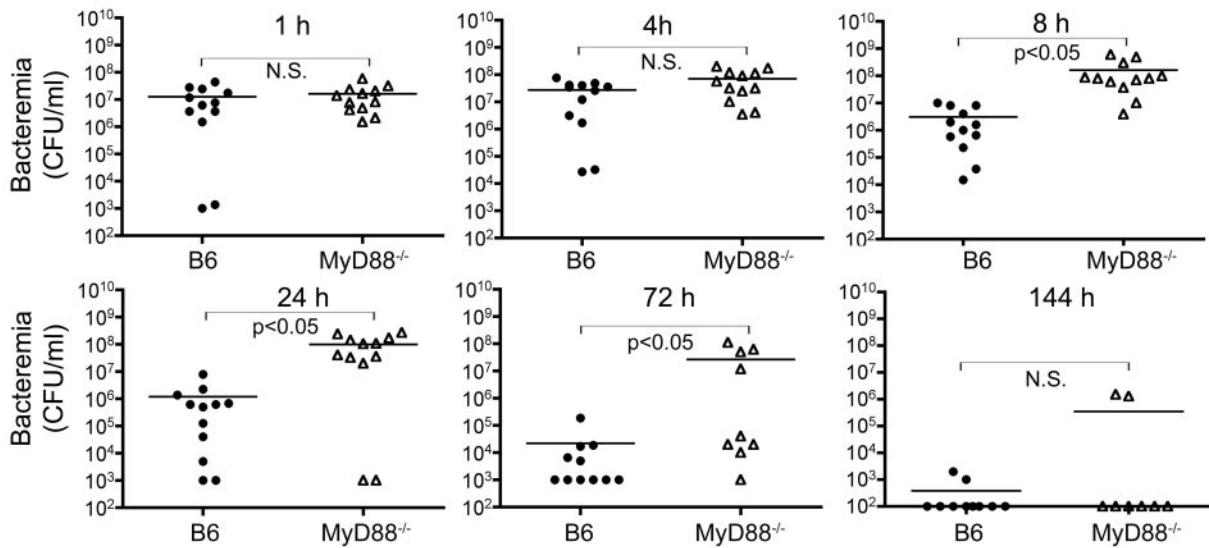
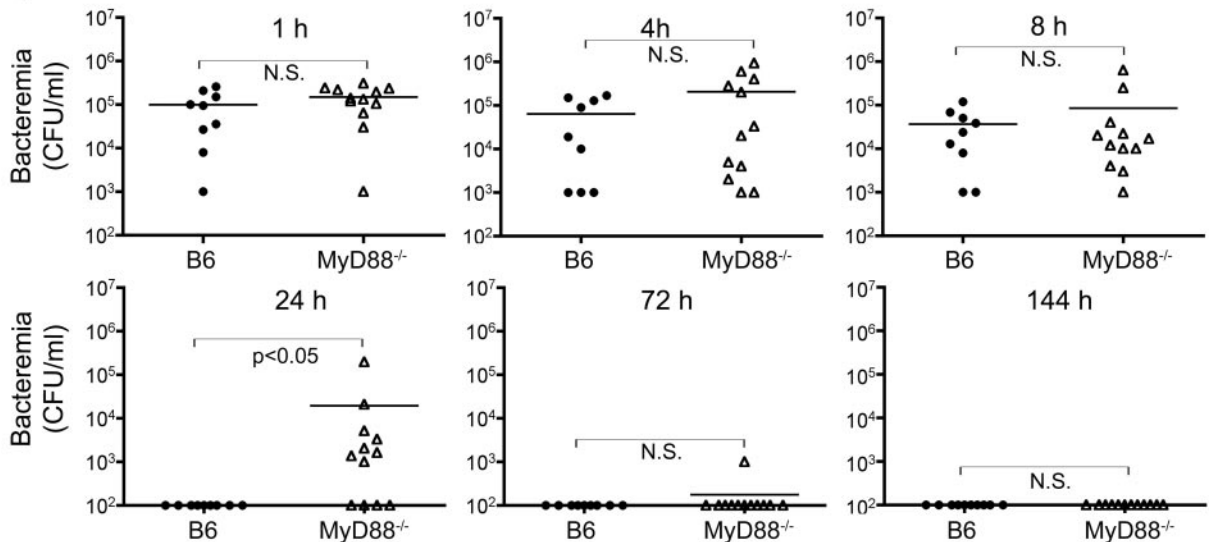
*lpxA*

FIG. 2. Bacteremia in mice infected with wild-type FAM20 or the *lpxA* mutant. Mice were infected with the wild-type bacterial strain FAM20 at 1×10^8 CFU or equivalent amounts of biological material of the *lpxA* mutant, and bacterial blood titers were monitored. All mice had similar bacteremia levels following infection with the *lpxA* mutant, except at 24 h postinfection ($P < 0.05$); however, bacteremia was significantly enhanced in *MyD88*^{-/-} mice following infection with the wild-type bacterial strain FAM20 ($P < 0.05$) at 8, 24, and 72 h postinfection. The counts were analyzed by the nonparametric Mann-Whitney test. N.S., nonsignificant difference.

MyD88^{-/-} mice infected intraperitoneally with *N. meningitidis* FAM20 and its LOS-deficient *lpxA* mutant was monitored (Fig. 1). Wild-type B6 mice and the *MyD88*^{-/-} mice infected with 1×10^8 CFU of FAM20/mouse had fatality rates of between 10 and 20%; however, the B6 mice showed signs of distress following infection, and this was not noted in the *MyD88*^{-/-} mice. Both mouse strains survived following infection with the LOS mutant at an equivalent amount of biological material as that given in the dose of 1×10^8 CFU of FAM20/mouse. At a higher dose of 5×10^8 CFU of FAM20/

mouse, or the equivalent amount of the *lpxA* mutant, significant fatality was noted in all B6 mice infected with the wild-type strain FAM20 and the *lpxA* mutant. In the *MyD88*^{-/-} mice, fatal infection occurred at 1 to 2 days postinfection in 50% of mice infected with FAM20. The *MyD88*^{-/-} mice were resistant to infection with the *lpxA* mutant. All subsequent assays were conducted in mice given a sublethal dose of 1×10^8 CFU/mouse of FAM20 or equivalent amounts of the *lpxA* mutant.

***MyD88*^{-/-} mice have higher bacteremia and delayed clearance of *N. meningitidis*.** Bacteremia levels were analyzed in

TABLE 1. Growth of meningococcal strains in whole mouse blood from B6 and MyD88^{-/-} mice

Time postinfection (h)	Bacterial strain	Growth in mouse blood (CFU/ml) ^a	
		B6	MyD88 ^{-/-}
1	FAM20 ^b	3.6 × 10 ⁵ (4.5 × 10 ⁴)	3.0 × 10 ⁵ (1.2 × 10 ⁴)
	<i>lpxA</i> mutant ^c	1.1 × 10 ⁵ (2.2 × 10 ⁴)	1.6 × 10 ⁵ (5.1 × 10 ⁴)
4	FAM20 ^b	4.6 × 10 ⁶ (1.4 × 10 ⁵)	3.6 × 10 ⁶ (4.3 × 10 ⁵)
	<i>lpxA</i> mutant ^c	3.6 × 10 ⁵ (1.2 × 10 ⁵)	8.0 × 10 ⁵ (1.4 × 10 ⁵)*

^a Data are presented as mean (standard deviation). *, statistically significant difference ($P < 0.05$) in bacterial numbers compared to numbers in B6 mice observed using a Student's *t* test.

^b Original inoculum, 1.8×10^5 CFU/ml.

^c Original inoculum, 1.1×10^5 CFU/ml.

mice administered the wild-type strain FAM20 or the LOS-deficient *lpxA* mutant (Fig. 2). An equivalent rate of entrance to the blood was noted in B6 and MyD88^{-/-} mice following infection with the wild-type bacterial strain FAM20, with no significant difference in bacteremia levels noted at 1 and 4 h postdose; however, at 8, 24, and 72 h postdose, the MyD88^{-/-} mice had a higher level of bacteremia, most likely related to the decreased capacity of these mice to clear the infection. At the early time points following administration of the *lpxA* mutant, no significant difference was noted in bacterial levels in blood of either mouse strain; however, at 24 h postinfection, a higher level of bacteremia was seen in the MyD88^{-/-} mice. In both mouse strains, the level of bacteremia following infection with FAM20 was between two- and threefold increased compared to the levels observed following infection with the *lpxA* mutant. The decreased clearance of the *lpxA* mutant in the MyD88^{-/-} mice correlated with increased bacterial survival in whole blood in vitro (Table 1). Significantly higher levels of the *lpxA* mutant were noted in MyD88^{-/-} mouse blood compared to B6 mouse blood at 4 h postinfection.

MyD88^{-/-} mice have lower proinflammatory cytokine levels following meningococcal infection. As expected, the levels of the proinflammatory cytokines IL-6 and TNF and the chemokine KC were lower in the MyD88^{-/-} mice than those seen in B6 mice (Fig. 3). In B6 mice, there was a significant increase in the level of IL-6 in mouse serum following infection with FAM20 only at 4 h postdose (Fig. 3A), which was not noted at other time points postdose, indicating that IL-6 induction is independent of LOS expression. In the MyD88^{-/-} mice, LOS deficiency resulted in a decreased IL-6 response at 4, 8, and 24 h postdose. The levels of the proinflammatory cytokine TNF (Fig. 3B) and the chemokine KC (Fig. 3C) were also lower in infected MyD88^{-/-} mice than in B6 mice, although no difference was noted between the wild-type strain FAM20 and the LOS-deficient *lpxA* mutant strain in either mouse strain, further confirming that another non-LOS factor is responsible for the proinflammatory effect in vivo. No statistical differences in basal levels of TNF or KC were noted in uninfected mice. The level of TNF in uninfected B6 mice was 4.1 ± 1.8 pg/ml, while the level in uninfected MyD88^{-/-} mice was 3.3 ± 0.8 pg/ml. The level of KC in uninfected B6 mice was 44.0 ± 30.3 pg/ml, while the level in uninfected MyD88^{-/-} mice was 93.9 ± 10.3 pg/ml.

MyD88^{-/-} mice have a decreased IL-10 response but increased levels of IFN- γ . IL-10 is an anti-inflammatory cytokine

(23, 36) with suppressive effects on the synthesis of proinflammatory cytokines and chemokines, such as TNF, IL-1 α , IL-1 β , IL-2, IL-6, and IL-8. IL-10 was not detected in uninfected mice. Following infection with FAM20, the IL-10 serum concentration in B6 mice was highest at 4 h postchallenge (Fig. 4). LOS-deficient bacteria triggered detectable IL-10 production only at 4 h postinfection, but IL-10 was undetectable at later time points. Sera from MyD88^{-/-} mice did not contain detectable levels of IL-10 after challenge with either bacterial strain, and these data indicate that MyD88^{-/-} mice have an impaired capacity to induce IL-10.

IL-10 is a strong inhibitor of IFN- γ production (26), and the

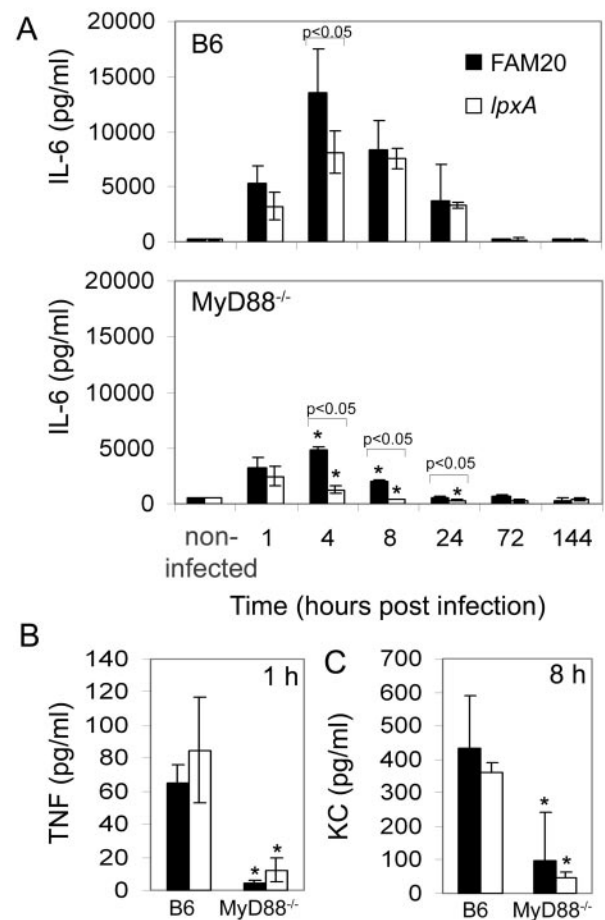


FIG. 3. The wild-type strain FAM20 (black bars) and the LOS-deficient *lpxA* mutant (white bars) induce similar levels of proinflammatory cytokines in serum of B6 and MyD88^{-/-} mice. (A) IL-6 induction in B6 and MyD88^{-/-} mice at various times postadministration (intraperitoneal) of 1×10^8 FAM20 cells/mouse or equivalent amounts of biological material of the *lpxA* mutant. (B) TNF induction was measured at 1 h postadministration, and (C) KC levels were monitored at 8 h postadministration in serum. Both TNF and KC were significantly decreased in MyD88^{-/-} mice; however, no difference was observed in the response to the wild-type strain FAM20 and the *lpxA* mutant. Statistically significant reductions of IL-6 compared to FAM20 are indicated by bars, and significance compared to the control B6 mice at each time point is indicated by a single asterisk when $P < 0.05$. Significance between treatments was determined using an unpaired Student's *t* test. Data are represented as means \pm standard deviation.

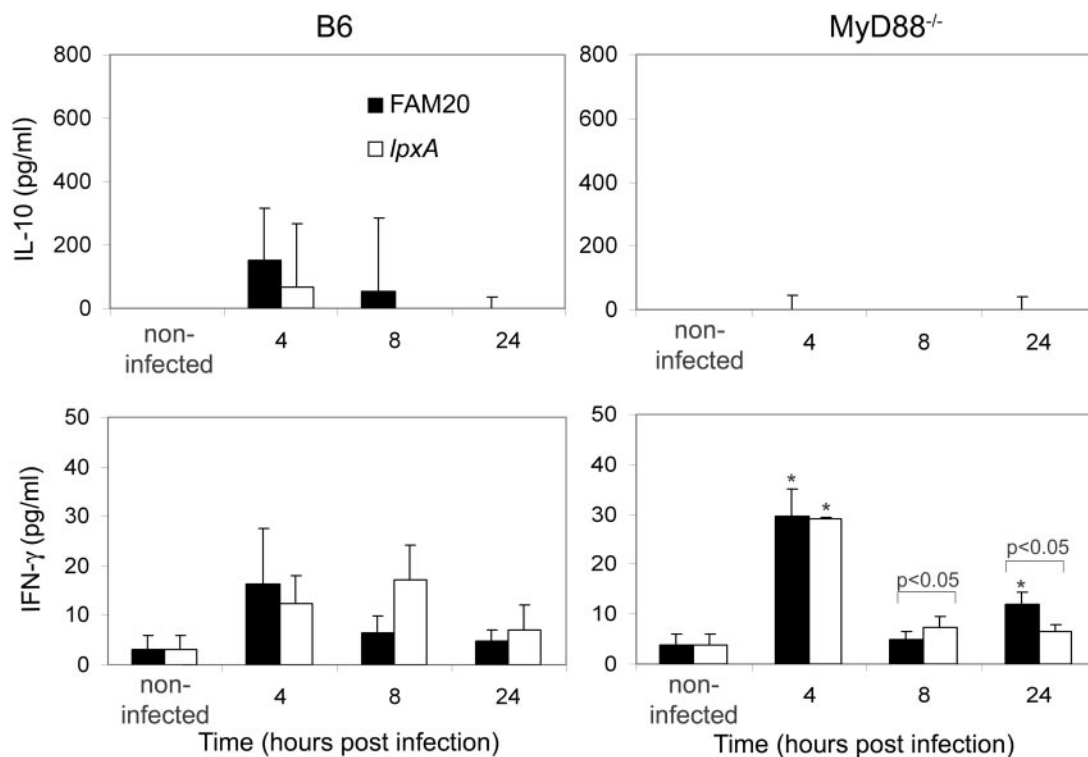


FIG. 4. The wild-type strain FAM20 (black bars) and the LOS-deficient mutant *lpxA* (white bars) induce similar levels of the regulatory cytokines IL-10 and IFN- γ in serum of B6 and MyD88^{-/-} mice. IL-10 levels were significantly increased in B6 mice at all time points postinfection compared to levels observed in MyD88^{-/-} mice following infection. IFN- γ was increased in MyD88^{-/-} mice at 4 h postinfection compared to levels in wild-type B6 mice. Statistically significant changes in mice infected with the *lpxA* mutant compared to FAM20 are indicated by bars. Significant increases are indicated with an asterisk when P is < 0.05 . Significance between treatments was determined using an unpaired Student's t test. Data are represented as means \pm standard deviation.

antagonistic effect of IL-10 on IFN- γ is reciprocal (14). IFN- γ enhances the function of macrophages and polymorphonuclear leukocytes by stimulating nonspecific defense mechanisms, such as phagocytosis and secretion of reactive oxygen intermediates (27). Uninfected B6 and MyD88^{-/-} mice had levels of IFN- γ of 3.0 ± 3.0 pg/ml and 3.8 ± 2.3 pg/ml, respectively. Both mouse strains showed similar levels of IFN- γ ; however, at 4 h postinfection, the levels induced in MyD88^{-/-} mice were significantly higher following administration of both bacterial strains (Fig. 4). We measured the phagocytosis of cells taken from the peritoneal cavity of mice and despite the induction of IFN- γ in MyD88^{-/-} mice, the peritoneal cells isolated from these mice showed inhibited phagocytosis of the *lpxA* mutant (data not shown).

Induction of the MyD88-independent cytokines IP-10 and IFN- β . We examined the production of cytokines IP-10 and IFN- β , which are induced via the MyD88-independent pathway. It has previously been shown that IP-10 can be induced by polymorphonuclear neutrophils following stimulation with meningococcal OM vesicles, but only if the neutrophils are stimulated in combination with IFN- γ (22). Uninfected B6 and MyD88^{-/-} mice had levels of IP-10 of 200.0 ± 19.2 pg/ml and 220.0 ± 108.0 pg/ml, respectively. IP-10 was induced in significantly larger amounts in B6 mice compared to in MyD88^{-/-} mice following infection with both strains (Fig. 5), despite the presence of higher levels of IFN- γ in the MyD88^{-/-} mice at

4 h postinfection (Fig. 4). No difference was observed in the level of IP-10 following infection with FAM20 or the LOS-deficient *lpxA* mutant, except for at 24 h postinfection in MyD88^{-/-} mice, where lower levels were observed following infection with the *lpxA* mutant. This further shows that in these mice the LOS of the wild-type bacterial strain has the potential to stimulate IP-10 production in a MyD88-independent manner. Interestingly, IFN- β was not significantly induced in either mouse strain following infection with either bacterial strain (data not shown).

MyD88^{-/-} mice show inhibited C5a induction. C5a is a potent chemotactic factor for neutrophils, monocytes, and macrophages, and production of C5a correlates with increased cytokine release and severe sepsis (15). The production of C5a in the serum of mice infected with FAM20 or the LOS-deficient mutant *lpxA* was monitored by ELISA analysis (Fig. 6). In the wild-type B6 mice, a sharp decrease in the level of C5a was noted at 1 h postinfection with both FAM20 and the LOS *lpxA* mutant. In mice infected with FAM20, the levels of C5a rose rapidly and C5a was seen at normal levels at all subsequent times. The level of C5a in the mice infected with the *lpxA* mutant did not rise to normal levels, and C5a was inhibited at all subsequent time points. In MyD88^{-/-} mice infected with FAM20, the sharp decrease observed in the wild-type mice at 1 h postdose was not noted and an increase in C5a levels was observed at 1 and 4 h postdose, indicating that LOS can stim-

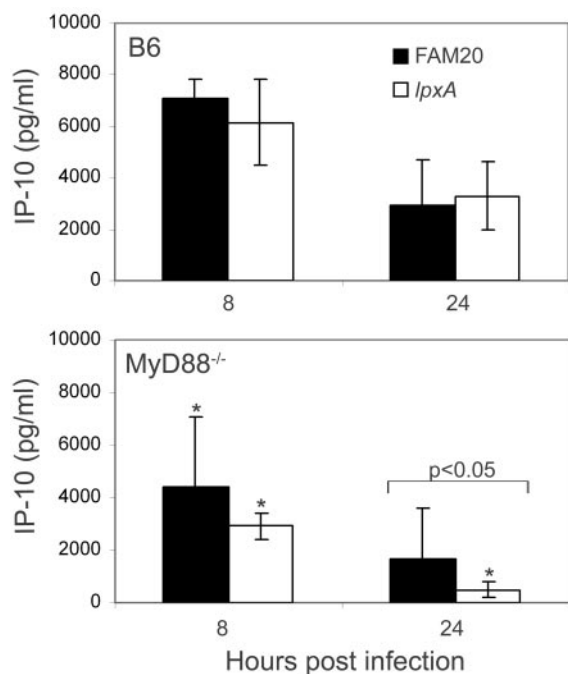


FIG. 5. Levels of IP-10 are decreased in MyD88^{-/-} mice. Statistically significant changes in mice infected with the *lpxA* mutant (white bars) compared to FAM20 (black bars) are indicated. Significantly decreased levels of these cytokines compared to the control B6 mice at each time point are indicated with a single asterisk when $P < 0.05$. Significance between treatments was determined using an unpaired Student's t test. Data are represented as means \pm standard deviation.

ulate C5a induction in the absence of MyD88. The basal levels of C5a in these mice were slightly lower than those in the wild-type mice, and the basal level was reached at 8 h postdose. The levels of C5a in MyD88^{-/-} mice infected with the *lpxA* mutant were significantly lower than those in the mice infected with FAM20 and in B6 mice at all time points.

MyD88 influences neutrophil infiltration in mice infected with the *lpxA* mutant. Polymorphonuclear neutrophils are natural effector cells mediating antimicrobial defenses via the release of toxic oxygen intermediates and lytic enzymes. The number of neutrophils as a percentage of total immune cells was determined by microscopic examination of blood smears. Both mouse strains showed similar neutrophil numbers prior to infection. Neutrophils constituted $23.8\% \pm 9.8\%$ and $20.3\% \pm 5.5\%$ of total blood cells in B6 mice and MyD88^{-/-} mice, respectively. In mice infected with FAM20, the infiltration of neutrophils into the blood was not affected by MyD88 (Fig. 7). Neutrophil infiltration in B6 mice following infection with the LOS mutant was inhibited, probably reflecting the lower bacteremia in these mice compared to that in mice infected with FAM20 at 24 h postdose. The neutrophil infiltration in MyD88^{-/-} mice infected with the *lpxA* mutant was significantly decreased at 24 h postinfection compared to infection in the B6 mice, as well as significantly decreased compared to infection with FAM20. This indicates that MyD88-dependent signaling is required for recognition of the LOS-deficient mutant and the recruitment of neutrophils to the blood.

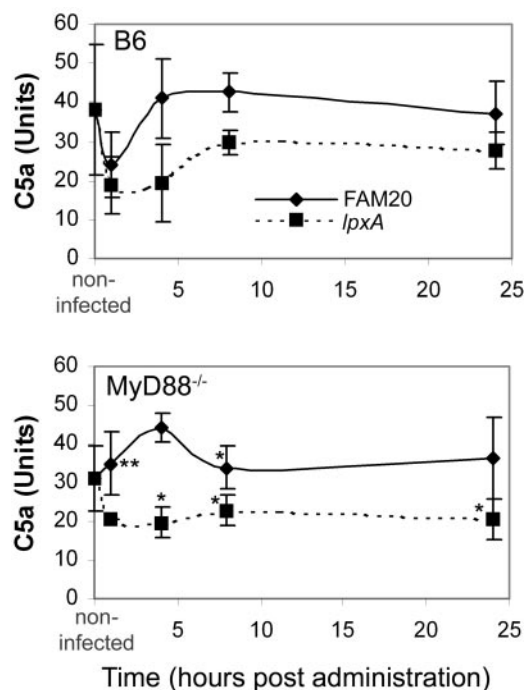


FIG. 6. Serum samples were assessed for the presence of the complement factor C5a from B6 and MyD88^{-/-} mice infected with FAM20 (solid lines) and the LOS-deficient *lpxA* mutant (dashed lines). Significance between treatments was determined using an unpaired Student's t test. Statistical decrease compared to treatment with FAM20 at each time point is indicated with a single asterisk when $P < 0.05$, while an increase is indicated with a double asterisk. Data are represented as means \pm standard deviation.

DISCUSSION

Sepsis caused by *Neisseria meningitidis* is a rapid and severe disease associated with high fatality rates. The fatality associated with sepsis has long been presumed to be dependent on the expression of LOS, and a correlation between disease severity and the levels of cytokines and circulating LOS has been observed (4). However, in this study we have observed that another neisserial factor is also involved in the development of septic shock, since an LOS-deficient mutant of the serogroup C strain FAM20 caused fatal sepsis and induced equivalent proinflammatory responses in the mouse model of meningococcal sepsis. We have also confirmed involvement of the adaptor MyD88 in the signaling pathway stimulated by this currently unidentified neisserial factor.

In contrast to other microbial diseases where the expression of various TLRs or adaptor or signaling molecules is considered to be beneficial and enhances clearance of the causative agent (21, 29, 45), expression of these factors in a mouse model of meningococcal sepsis is associated with fatality. It is unlikely that development of sepsis is a direct result of responses to the bacteria itself, since both strains showed similar levels of fatality in the wild-type B6 mice despite vastly different levels of bacteremia. It is more likely that the excessive production of proinflammatory cytokines, chemokines, and costimulatory and adhesion molecules or the generation of heat shock proteins contributes to disease severity.

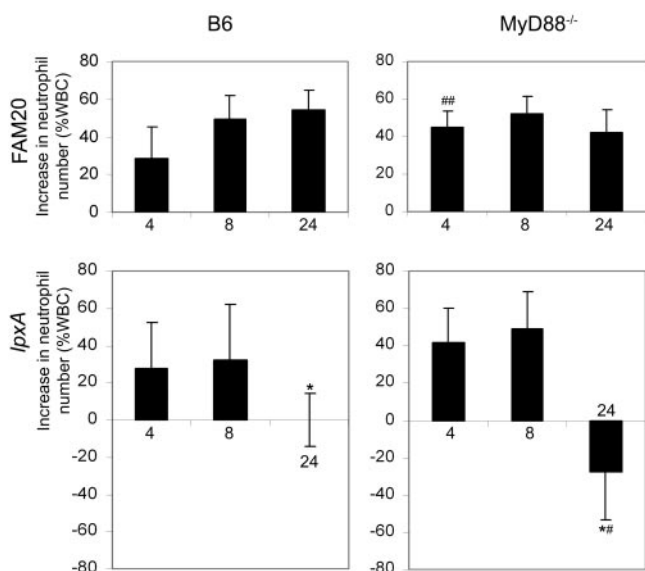


FIG. 7. Infiltration of neutrophils into blood of B6 and MyD88^{-/-} mice infected with FAM20 and the LOS-deficient *lpxA* mutant. Significant decreases in neutrophil levels in MyD88^{-/-} mice compared to B6 mice were determined using an unpaired Student's *t* test, and statistically reduced ($P < 0.05$) neutrophil levels are indicated with #, while significant inductions are indicated with ##. Statistically significant decreases compared to FAM20 infection are indicated with a single asterisk when $P < 0.05$. Data are represented as means \pm standard deviation. WBC, white blood cells.

In this study, we observed that MyD88^{-/-} mice showed resistance to meningococcal killing, indicating that signaling via MyD88 is associated with enhanced morbidity and mortality in this mouse model and is involved in the development of sepsis. MyD88^{-/-} mice had a decreased capacity to produce the proinflammatory cytokines IL-6 and TNF and the chemokine KC and had lower C5a levels and decreased neutrophils in the blood when infected with the LOS-deficient *lpxA* mutant. The cytokines IL-6 and TNF and the chemokine KC are under control of the NF- κ B promoter. These cytokines are all induced early in a MyD88-dependent manner; however, since NF- κ B is induced later in response to the IRF-3 adaptor, a late induction of these cytokines could also be seen. It has previously been shown that a meningococcal serogroup B LOS mutant shows poor activation of NF- κ B (9), which is inconsistent with our data showing that both meningococcal strains induced equivalent amounts of these cytokines. Also, other *in vitro* studies using cell models of monocytes (40, 43), differentiated macrophages (32, 46), dendritic cells (10), and meningeal cells (16) have shown that meningococci deficient in LOS are able to induce significant cellular activation, although levels of cytokines induced in most of these studies were reduced compared to those observed following infection with the parent strain.

A recent study by Zughaier et al. (47) identified IFN- α/β as key molecules in the differential induction of the MyD88-independent signaling pathway to *N. meningitidis* LOS. We did not observe an increase in the level of IFN- β in serum from mice infected with either the wild-type strain FAM20 or the LOS-deficient *lpxA* mutant. It is possible that IFN- α is respon-

sible for the effects or that low concentrations of IFN- β that were not detectable in the ELISA analysis affect signaling. IP-10 is an important chemokine involved in T-cell recruitment. It is produced in response to IFN- γ in combination with either TNF or bacterial lipopolysaccharides, and the response is blocked by IL-10 and IL-4 (11). LOS is not the only ligand responsible for the induction of IP-10 *in vivo*, since the wild-type strain and the *lpxA* mutant both induced equivalent IP-10 levels at 8 h postinfection (Fig. 5). However, at 24 h postinfection, the level of IP-10 was significantly reduced following infection with the LOS mutant in the MyD88^{-/-} mice, confirming previous findings that LOS stimulates IP-10 production in the absence of MyD88-dependent signaling (19). However, our findings also confirm that MyD88 also plays a role in the induction of IP-10 (28) since levels were decreased in MyD88^{-/-} mice compared to B6 mice.

In this study, we have also observed an involvement of C5a in the innate response to LOS-deficient meningococcal infection (Fig. 6). We showed a decrease in the levels of C5a in the serum of mice infected with the LOS-deficient meningococcal strain. This was surprising since it had previously been determined that complement activation and the subsequent inflammation are induced by factors independent of meningococcal lipooligosaccharide (38, 39). The decrease of C5a was significantly pronounced in MyD88^{-/-} mice, indicating that the C5a response to the LOS-deficient meningococcal strain is mediated via MyD88. Decreased C5a levels in MyD88^{-/-} mice could also account for the reduced neutrophil numbers in these mice. Antibodies to C5a have been shown to be important in the prevention of meningococcal sepsis (37) and provide an explanation for the difference in disease severity between B6 mice and MyD88^{-/-} mice.

High levels of neutrophils were shown to infiltrate into the blood circulation in both B6 and MyD88^{-/-} mice following infection, although the *lpxA* mutant had a decreased ability to cause neutrophil infiltration compared to the wild-type strain (Fig. 7). At 24 h, neutrophil levels were not related to bacteremia levels, which were significantly higher in MyD88^{-/-} mice following administration of both bacterial strains at this time (Fig. 2). It is likely that the decrease in neutrophil levels is a direct result of the absence of unmeasured chemotactic factors in the blood. The decreased responses in MyD88^{-/-} mice also indicate that although neutrophils are present in the blood, the function of these cells may be compromised.

Lipooligosaccharide of meningococci has previously been shown to have a central role in the development of sepsis in humans (5–7), and the response to LOS is primarily mediated via the TLR4-dependent signaling pathway (2, 46). The findings of this study indicate that meningococci express a non-LOS ligand that makes a significant contribution to the development of meningococcal septic shock. Although the neisserial porin has been shown to be a TLR2 ligand which signals via the MyD88 pathway (24, 35), the fatality associated with the *lpxA* mutant described here is not due to the action of porin. This is evident from unpublished data showing that infection of TLR2^{-/-} mice with the *lpxA* mutant results in sepsis equivalent to that observed in wild-type mice.

In this study, we have used an *in vivo* animal model to study the effects of meningococcal LOS. We have noted that MyD88^{-/-} mice are not susceptible to LOS-deficient menin-

gococcal infection despite increased bacteremia levels and decreased innate immunity, showing that the response to an unidentified neisserial PAMP occurs in a MyD88-dependent manner. Identification of the unknown ligand is of critical importance in understanding the development of meningococcal sepsis.

ACKNOWLEDGMENTS

We thank S. Akira, K. Takeda, and O. Takeuchi, Department of Host Defense, Research Institute for Microbial Diseases, Osaka, Japan, for the MyD88^{-/-} mice.

This work was supported by grants from the Swedish Medical Research Council (Dnr 2004-4831 and 2002-6240), Swedish Cancer Society, Swedish Society for Medicine, Wenner-Gren Foundations, and Uppsala University.

REFERENCES

- Albiger, B., L. Johansson, and A. B. Jonsson. 2003. Lipooligosaccharide-deficient *Neisseria meningitidis* shows altered pilus-associated characteristics. *Infect. Immun.* **71**:155–162.
- Bjerrre, A., B. Brusletto, R. Ovstebo, G. B. Joo, P. Kierulf, and P. Brandtzaeg. 2003. Identification of meningococcal LPS as a major monocyte activator in IL-10 depleted shock plasmas and CSF by blocking the CD14-TLR4 receptor complex. *J. Endotoxin Res.* **9**:155–163.
- Bonnah, R. A., J. Hoelter, L. Steeghs, C. A. Enns, M. So, and M. U. Muckenthaler. 2005. Lipooligosaccharide-independent alteration of cellular homeostasis in *Neisseria meningitidis*-infected epithelial cells. *Cell Microbiol.* **7**:869–885.
- Brandtzaeg, P., A. Bjerrre, R. Ovstebo, B. Brusletto, G. B. Joo, and P. Kierulf. 2001. *Neisseria meningitidis* lipopolysaccharides in human pathology. *J. Endotoxin Res.* **7**:401–420.
- Brandtzaeg, P., K. Bryn, P. Kierulf, R. Ovstebo, E. Namork, B. Aase, and E. Jantzen. 1992. Meningococcal endotoxin in lethal septic shock plasma studied by gas chromatography, mass-spectrometry, ultracentrifugation, and electron microscopy. *J. Clin. Investig.* **89**:816–823.
- Brandtzaeg, P., P. Kierulf, P. Gaustad, A. Skulberg, J. N. Bruun, S. Halvorsen, and E. Sorensen. 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. *J. Infect. Dis.* **159**:195–204.
- Brandtzaeg, P., T. E. Mollnes, and P. Kierulf. 1989. Complement activation and endotoxin levels in systemic meningococcal disease. *J. Infect. Dis.* **160**:58–65.
- Claus, H., M. C. Maiden, D. J. Wilson, N. D. McCarthy, K. A. Jolley, R. Urwin, F. Hessler, M. Frosch, and U. Vogel. 2005. Genetic analysis of meningococci carried by children and young adults. *J. Infect. Dis.* **191**:1263–1271.
- Dixon, G. L., R. S. Heyderman, P. van der Ley, and N. J. Klein. 2004. High-level endothelial E-selectin (CD62E) cell adhesion molecule expression by a lipopolysaccharide-deficient strain of *Neisseria meningitidis* despite poor activation of NF- κ B transcription factor. *Clin. Exp. Immunol.* **135**:85–93.
- Dixon, G. L., P. J. Newton, B. M. Chain, D. Katz, S. R. Andersen, S. Wong, P. van der Ley, N. Klein, and R. E. Callard. 2001. Dendritic cell activation and cytokine production induced by group B *Neisseria meningitidis*: interleukin-12 production depends on lipopolysaccharide expression in intact bacteria. *Infect. Immun.* **69**:4351–4357.
- Dufour, J. H., M. Dziejman, M. T. Liu, J. H. Leung, T. E. Lane, and A. D. Luster. 2002. IFN- γ -inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J. Immunol.* **168**:3195–3204.
- Dyer, D. W., W. McKenna, J. P. Woods, and P. F. Sparling. 1987. Isolation by streptonigrin enrichment and characterization of a transferrin-specific iron uptake mutant of *Neisseria meningitidis*. *Microb. Pathog.* **3**:351–363.
- Fisette, P. L., S. Ram, J. M. Andersen, W. Guo, and R. R. Ingalls. 2003. The Lip lipoprotein from *Neisseria gonorrhoeae* stimulates cytokine release and NF- κ B activation in epithelial cells in a Toll-like receptor 2-dependent manner. *J. Biol. Chem.* **278**:46252–46260.
- Florquin, S., Z. Amraoui, D. Abramowicz, and M. Goldman. 1994. Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice. *J. Immunol.* **153**:2618–2623.
- Guo, R. F., and P. A. Ward. 2005. Role of C5a in inflammatory responses. *Annu. Rev. Immunol.* **23**:821–852.
- Humphries, H. E., M. Triantafyllou, B. L. Makepeace, J. E. Heckels, K. Triantafyllou, and M. Christodoulides. 2005. Activation of human meningeal cells is modulated by lipopolysaccharide (LPS) and non-LPS components of *Neisseria meningitidis* and is independent of Toll-like receptor (TLR)4 and TLR2 signalling. *Cell Microbiol.* **7**:415–430.
- Ingalls, R. R., E. Lien, and D. T. Golenbock. 2000. Differential roles of TLR2 and TLR4 in the host response to Gram-negative bacteria: lessons from a lipopolysaccharide-deficient mutant of *Neisseria meningitidis*. *J. Endotoxin Res.* **6**:411–415.
- Kahler, C. M., and D. S. Stephens. 1998. Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). *Crit. Rev. Microbiol.* **24**:281–334.
- Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* **167**:5887–5894.
- Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and D. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* **85**:1274–1279.
- Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H. W. Pfister, and C. J. Kirschning. 2003. Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. *J. Immunol.* **170**:438–444.
- Lapinet, J. A., P. Scapini, F. Calzetti, O. Peréz, and M. A. Cassatella. 2000. Gene expression and production of tumor necrosis factor alpha, interleukin-1 β (IL-1 β), IL-8, macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , and gamma interferon-inducible protein 10 by human neutrophils stimulated with group B meningococcal outer membrane vesicles. *Infect. Immun.* **68**:6917–6923.
- Marchant, A., M. L. Alegre, A. Hakim, G. Pierard, G. Marecaux, G. Friedman, D. De Groote, R. J. Kahn, J. L. Vincent, and M. Goldman. 1995. Clinical and biological significance of interleukin-10 plasma levels in patients with septic shock. *J. Clin. Immunol.* **15**:266–273.
- Massari, P., W. Henneke, Y. Ho, E. Lutz, D. T. Golenbock, and L. M. Wetzler. 2002. Cutting edge: immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. *J. Immunol.* **168**:1533–1537.
- Mirlashari, M. R., and T. Lyberg. 2003. Expression and involvement of Toll-like receptors (TLR)2, TLR4, and CD14 in monocyte TNF- α production induced by lipopolysaccharides from *Neisseria meningitidis*. *Med. Sci. Monit.* **9**:BR316–BR324.
- Moore, K., A. O'Garra, R. De Waal Malefyt, P. Viera, and T. R. Mosmann. 1993. Interleukin-10. *Annu. Rev. Immunol.* **11**:165–190.
- Murray, J. W. 1981. Susceptibility of Leishmania to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* **153**:1302–1315.
- Nagarajan, U. M., D. M. Ojcius, L. Stahl, R. G. Rank, and T. Darville. 2005. Chlamydia trachomatis induces expression of IFN- γ -inducible protein 10 and IFN- β independent of TLR2 and TLR4, but largely dependent on MyD88. *J. Immunol.* **175**:450–460.
- Naiki, Y., K. S. Michelsen, N. W. Schroder, R. Alsabeh, A. Slepkin, W. Zhang, S. Chen, B. Wei, Y. Bulut, M. H. Wong, E. M. Peterson, and M. Arditi. 2005. MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of Chlamydia pneumoniae pneumonia. *J. Biol. Chem.* **280**:29242–29249.
- Neal, K. R., J. S. Nguyen-Van-Tam, N. Jeffrey, R. C. Slack, R. J. Madeley, K. Ait-Tahar, K. Job, M. C. Wale, and D. A. Ala'Aldeen. 2000. Changing carriage rate of *Neisseria meningitidis* among university students during the first week of term: cross sectional study. *Br. Med. J.* **320**:846–849.
- Peiser, L., M. P. J. De Winther, K. Makepeace, M. Hollinshead, P. Coull, J. Plested, T. Kodama, E. R. Moxon, and S. Gordon. 2002. The class A macrophage scavenger receptor is a major pattern recognition receptor for *Neisseria meningitidis* which is independent of lipopolysaccharide and not required for secretory responses. *Infect. Immun.* **70**:5346–5354.
- Pridmore, A. C., D. H. Wyllie, F. Abdillahi, L. Steeghs, P. van der Ley, S. K. Dower, and R. C. Read. 2001. A lipopolysaccharide-deficient mutant of *Neisseria meningitidis* elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. *J. Infect. Dis.* **183**:89–96.
- Rahman, M., H. Kallstrom, S. Normark, and A. B. Jonsson. 1997. PilC of pathogenic *Neisseria* is associated with the bacterial cell surface. *Mol. Microbiol.* **25**:11–25.
- Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahring, U. Seydel, F. Di Padova, et al. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* **8**:217–225.
- Singleton, T. E., P. Massari, and L. M. Wetzler. 2005. Neisserial porin-induced dendritic cell activation is MyD88 and TLR2 dependent. *J. Immunol.* **174**:3545–3550.
- Spits, H., and R. de Waal. 1992. Functional characterization of human IL-10. *Int. Arch. Allergy Immunol.* **99**:8–15.
- Sprong, T., P. Brandtzaeg, M. Fung, A. M. Pharo, E. A. Hoiby, T. E. Michaelsen, A. Aase, J. W. van der Meer, M. van Deuren, and T. E. Mollnes. 2003. Inhibition of C5a-induced inflammation with preserved C5b-9-mediated bactericidal activity in a human whole blood model of meningococcal sepsis. *Blood* **102**:3702–3710.
- Sprong, T., D. L. Jack, N. J. Klein, M. W. Turner, P. van der Ley, L. Steeghs, L. Jacobs, J. W. van der Meer, and M. van Deuren. 2004. Mannose binding

- lectin enhances IL-1b and IL-10 induction by non-lipopolysaccharide (LPS) components of *Neisseria meningitidis*. *Cytokine* **28**:59–66.
39. **Sprong, T., A.-S. Moller, A. Bjerre, E. Wedege, P. Kierulf, J. W. van der Meer, P. Brandtzaeg, M. van Deuren, and T. E. Mollnes.** 2004. Complement activation and complement-dependent inflammation by *Neisseria meningitidis* are independent of lipopolysaccharide. *Infect. Immun.* **72**:3344–3349.
40. **Sprong, T., N. Stikkelbroeck, P. van der Ley, L. Steeghs, L. van Alphen, N. Klein, M. G. Netea, J. W. van der Meer, and M. van Deuren.** 2001. Contributions of *Neisseria meningitidis* LPS and non-LPS to proinflammatory cytokine response. *J. Leukoc. Biol.* **70**:283–288.
41. **Takeda, K., and S. Akira.** 2004. TLR signaling pathways. *Semin. Immunol.* **16**:3–9.
42. **Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono.** 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu. Rev. Immunol.* **23**:307–336.
43. **Uronen, H., A. J. Williams, G. Dixon, S. R. Andersen, P. Van Der Ley, M. Van Deuren, R. E. Callard, and N. Klein.** 2000. Gram-negative bacteria induce proinflammatory cytokine production by monocytes in the absence of lipopolysaccharide (LPS). *Clin. Exp. Immunol.* **122**:312–315.
44. **van der Ley, P., and L. Steeghs.** 2003. Lessons from an LPS-deficient *Neisseria meningitidis* mutant. *J. Endotoxin Res.* **9**:124–128.
45. **Wieland, C. W., S. Florquin, N. A. Maris, K. Hoebe, B. Beutler, K. Takeda, S. Akira, and T. van der Poll.** 2005. The MyD88-dependent, but not the MyD88-independent, pathway of TLR4 signaling is important in clearing nontypeable *Haemophilus influenzae* from the mouse lung. *J. Immunol.* **175**:6042–6049.
46. **Zughaier, S. M., Y.-L. Tzeng, S. M. Zimmer, A. Datta, R. W. Carlson, and D. S. Stephens.** 2004. *Neisseria meningitidis* lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway. *Infect. Immun.* **72**:371–380.
47. **Zughaier, S. M., S. M. Zimmer, A. Datta, R. W. Carlson, and D. S. Stephens.** 2005. Differential induction of the Toll-like receptor 4–MyD88-dependent and -independent signaling pathways by endotoxins. *Infect. Immun.* **73**:2940–2950.

Editor: J. N. Weiser