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*TLR2 and Caspase-8 Are Essential for Group B Streptococcus-Induced Apoptosis in Microglia*

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TLR2 and Caspase-8 Are Essential for Group B Streptococcus-Induced Apoptosis in Microglia


Microglia, the resident innate immune cells of the CNS, detect invading pathogens via various receptors, including the TLR. Microglia are involved in a number of neurodegenerative diseases in which their activation may be detrimental to neurons. It is largely unknown how this potentially deleterious action can be countered on a cellular level. We previously found that the interaction of TLR2 with group B Streptococcus (GBS), the most important pathogen in neonatal bacterial meningitis, activates microglia that in turn generate neurotoxic NO. We report in this study that GBS not only activates microglia, but also induces apoptosis in these cells via TLR2 and the TLR-adaptor molecule MyD88. Soluble toxic mediators, such as NO, are not responsible for this form of cell death. Instead, interaction of GBS with TLR2 results in formation and activation of caspase-8, a process that involves the transcription factor family Ets. Whereas caspase-8 plays an essential role in GBS-induced microglial apoptosis, caspase-3 is dispensable in this context. We suggest that TLR2- and caspase-8-mediated microglial apoptosis constitutes an autoregulatory mechanism that limits GBS-induced overactivation of the innate immune system in the CNS. The Journal of Immunology, 2007, 179: 6134–6143.

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roup B Streptococcus (GBS, or Streptococcus agalactiae) accounts for ~50% of all cases of neonatal meningitis (1) and represents the third most frequent cause of bacterial meningitis overall (2, 3). Bacterial meningitis is most commonly a manifestation of late-onset disease, which occurs after the first week of life and currently accounts for up to 50% of all neonatal cases of invasive GBS infections (4). GBS-induced meningitis is associated with long-term neurological and developmental morbidity, including cognitive impairment, language disorders, seizures, and motor handicaps, in up to 52% of surviving infants (5, 6). The molecular mechanisms underlying the damage to the immature brain are largely unresolved.

Microglia represent the resident immune host defense and are considered the major inflammatory cell type in the CNS. In CNS infection, microglia play an ambiguous role. On the one hand, microglia sense substructures of various pathogens and orchestrate the antimicrobial immune response that protects the brain against invading organisms. In contrast, activated microglia have been implicated in neurodegeneration resulting from bacterial meningitis, AIDS dementia complex, and multiple sclerosis, or associated with inflammation such as Parkinson’s disease or Alzheimer’s disease (7–10). Upon activation, microglia secrete several proinflammatory and cytotoxic molecules, including ILs, TNF-α, reactive oxygen species such as NO, eicosanoids, and arachidonic acid metabolites (11). Accordingly, we have previously found that GBS potently induces secretion of NO in primary mouse microglia (12). An insufficient microglia-triggered inflammatory response that fails to prevent spread of pathogens such as GBS may have a potentially deleterious impact on neurons. In contrast, a sustained and therefore uncontrolled production of toxic products released from microglia may itself cause irreversible damage to neurons because neuronal tissue is not capable of self-regeneration. Thus, it is conceivable that via evolutionary forces the human brain has developed strategies to evade damage inflicted by its own local immune system. B and T lymphocytes undergo activation-induced cell death (AICD) that enables the organism to limit inflammatory processes (13, 14). Similar mechanisms have recently been described for macrophages and microglia (15, 16). Several pathogens including GBS induce apoptosis in macrophages to combat host immune defense (17, 18). In accordance with this, microglia and astrocytes undergo apoptosis upon inflammatory activation (19, 20). However, the molecular mechanisms that trigger and control bacteria-induced AICD in the CNS remain unclear.

We recently showed that GBS activates microglia via TLR2 and consequently leads to neuronal injury in vitro (12). TLRs recognize invariant molecular structures associated with pathogens and initiate an innate immune response (21). To date, 13 TLR orthologues, of which 10 are expressed in humans, have been identified. The best characterized pathway downstream of TLRs involves the intracellular proteins MyD88, IL-1R-associated kinases 1–4, and TNFR-associated factor 6. Activation of these molecules ultimately results in the activation of the transcriptional factor NF-κB (22). TLR2 recognizes several Gram-positive bacteria as well as the bacterial cell wall components peptidoglycan and lipoteichoic acid (LTA), and its activation results in the production of cytokines.
GBS induces apoptosis in microglia. In contrast to neuronal cell death triggered by GBS-activated microglia, apoptosis in microglia itself occurs independently of NO or other molecules secreted from microglia. GBS induces caspase-8, but not caspase-3, via TLR2. The Ets family of transcription factors is essential in this context. Accordingly, a functional TLR2 pathway is crucial not only for activation of microglia, but is also required for cell death and therefore silencing of microglia in response to the same organism.

Materials and Methods

Animals
MyD88-/- and TLR2-/- mice (29, 30) were provided by S. Akira, Department of Host Defense, Osaka University (Osaka, Japan). B6Smn.C3-Fas(+/−)/J (Fas ligand [FasL]-deficient/gld) and C57BL/6J mice were purchased from The Jackson Laboratory. All experimental procedures were conducted in accordance with the guidelines of the Charite animal facility and were approved by the local ethics committee.

Generation of heat-fixed (HF)-GBS and live GBS, HF-Streptococcus pneumoniae, and LTA
The GBS type III strains COH1 and NEM316, both of which were originally isolated from newborn infants with sepsis, the NEM316-derivative NEM1636, and S. pneumoniae (D39 capsular type 2) were cultured and heat fixed, as previously described (24, 31, 32). GBS NEM316 and 1636 were gifts of Patrick Trieu-Couss (Paris, France). Streptococcus aureus LTA was obtained from InvivoGen, and GBS LTA was prepared as previously described (24). Live GBS COH1 was maintained and grown in Todd-Hewitt broth (BD Diagnostics) as standing cultures at 37°C until the mid-log phase (−106 CFU/ml; OD600 = 0.4), at which point the bacteria were harvested, centrifuged, and resuspended in DMEM (Invitrogen Life Technologies) in indicated concentrations. HF-GBS was used throughout this study, unless otherwise indicated. In the case of HF-GBS, the concentrations are given as the number of bacteria equivalent to CFU, determined immediately before heat inactivation (as determined on blood agar).

Primary cultures of purified microglia
Purified microglia were generated from forebrains of P0-3 mice, as previously described (33). Brieﬂy, brain tissue was dissociated with trypsin (Invitrogen Life Technologies) for 20 min at 37°C. After mechanical dissociation, cells were plated in 75-cm2 culture ﬂasks in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS and penicillin/streptomycin. After 10 days in culture, mixed glial cultures were shaken for 30 min at 180 rpm. The supernatant containing >97% microglia was plated on poly-L-lysine (BD Biosciences)-coated glass coverslips. Purified microglial cells were maintained in DMEM with 10% FBS at 37°C.

Cell staining
For isoelectric B4 (IB4) staining, live cultures were incubated for 30 min with IB4 (Molecular Probes), washed with PBS, then ﬁxed in fresh 4% paraformaldehyde in PBS for 10 min at ambient temperature and washed with PBS. For 4′-diamino-2-phenylindole (DAPI) staining, cells were ﬁxed with 4% paraformaldehyde and then incubated with DAPI (Molecular Probes) for 2 min. TUNEL staining of microglia was conducted using the In Situ Death Detection Kit, TMR red, according to the instruction manual (Roche). Caspase-8 staining was performed using the Image-iT LIVE Green Caspase-8 Detection kit, following the instruction manual (Molecular Probes). Finally, the cells were visualized by epifluorescence.

Measurement of nitrite
GBS-induced production of NO in microglia was analyzed by measuring the stable end product nitrite in the culture supernatants. The amount of NO3− was determined by using the colorimetric Griess reaction (Sigma-Aldrich), as described previously (34). The inducible NO synthase inhibitor aminoguanidine was also obtained from Sigma-Aldrich.

Western blotting
Cells were washed with PBS and lysed on ice in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) with 10 mg/ml protease inhibitor mixture (Sigma-Aldrich). The protein extracts were boiled for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was blocked with 10% nonfat milk in PBS for 1 h at room temperature and incubated with an Ab directed against caspase-3 (1:100; Chemicon International) at 4°C overnight. Equal loading of protein was ensured by parallel probing with an Ab directed against β-actin. Subsequently, the membrane was washed 3 × 5 min with 0.05% Tween 20 in PBS and then incubated with the appropriate secondary Ab (Amersham) for 1 h. After another round of washing, precipitates were visualized by ECL detection.

Reporter gene analysis
The human hepatoma cell line HuH7 was used in the reporter gene studies because it best supported the caspase-8 promoter. RT-PCR revealed weak, but reproducible transcription of TLR2. HuH7 cells were plated into 96-well tissue culture plates at a density of 5 × 104 cells/well. The following day, cells were transiently transfected with TransIT-LT1 Transfection Reagents (Mirus Bio) following the manufacturer’s protocol. The following luciferase reporter constructs were used: a bp−470/−76 construct of the caspase-8 promoter that has previously been shown to potently mediate transcriptional activation of the gene, and the −470/−76 caspase-8 construct containing a point mutation in an Ets-like element binding site (∆ets) (35). In addition, cells were transfected with human TLR2-yellow fluorescent protein (36), as indicated. pcDNA was used to assure equal amounts of transfected DNA. The following day, cells were stimulated for 24 h, as indicated. Cells were then lysed in passive lysis buffer (Promega), and reporter gene activity was measured using a plate reader luminometer (MicroLumat Plus; Berthold Detection Systems).

Cytotoxicity experiments and statistical analysis
For experiments using HF-GBS, microglia were seeded at a density of 35,000 cells/well (96-well plates) or 120,000 cells/well (24-well plates). On the day of the experiment, the medium of the microglial cultures was completely replaced by 200 or 500 μl of fresh medium, respectively, and cultures were treated with 108 HF-GBS COH1/ml, unless noted otherwise. For experiments using live GBS, microglia were seeded at a density of 35,000 cells/well (96-well plates) and were incubated with 104 or 105 CFU live GBS/ml (106 CFU live GBS/ml are equivalent to a multiplicity of infection of 5). GBS LTA or S. aureus LTA were used in the indicated concentrations. After the caspase inhibitor assay, 100 μM Z-VAD-FMK, 100 μM Z-IETD-FMK, or 120 μM DEVD-CHO (Calbiochem) was added 2 h before GBS treatment. Cultlase (1250 U/ml) and FasL were purchased from Sigma-Aldrich. For experiments using Transwell chambers, Millicell cell culture inserts (pore size 0.4 μm; Millipore) were used.

The figures show representative results from three to six independent experiments, each using triplicate wells. Data are expressed as mean ± SD. Statistical analysis was performed with SigmaStat version 2.03 (SPSS) using Student’s t test.

Results
HF-GBS induces apoptosis in microglia
To investigate cytotoxic effects of GBS on microglia, microglia derived from cortices of wild-type mice were incubated with HF-GBS, and the effect was visualized by phase-contrast microscopy (Fig. 1A). In purified microglial cultures, HF-GBS induced a signiﬁcant reduction of the cell number compared with control conditions. Next, we determined whether the cell death observed after incubation with HF-GBS was due to apoptosis. GBS-treated cell cultures were labeled with the isoelectric IB4 to identify microglia, and then analyzed by TUNEL assay (Fig. 1B). Upon stimulation with HF-GBS, 95.1% of IB4-positive cells underwent apoptosis as indicated by TUNEL assay. Parallel immunostaining with the astrocyte-specific Ab glial fibrillary acidic protein demonstrated a purity of 97% for microglia in isolated glial cultures (data not shown). Staining with DAPI confirmed the HF-GBS-induced cell death by revealing morphological hallmarks of apoptosis, such as condensation, shrinkage, and fragmentation of the nuclei (Fig. 1C). DAPI staining did not only label the nuclei of microglia, but also stained, and therefore visualized, internalized HF-GBS in these cells.
Dose-response experiments and subsequent quantitation of surviving IB4-positive cells showed a significant reduction in relative microglial viability after incubation with $10^7$–$10^9$ HF-GBS/ml for 72 h (Fig. 1D). Incubation of microglia with $10^8$ HF-GBS/ml for various incubation times led to a significant reduction in relative microglial viability after as soon as 48 h (Fig. 1E).

To test for the specificity of the used pathogen in terms of microglial toxicity, another Gram-positive bacteria, *S. pneumoniae*, which represents the primary cause of bacterial meningitis in children after the age of 3 mo, was analyzed for cytotoxic properties in microglial cultures. To this end, purified wild-type microglia were incubated at various doses (Fig. 1F) for various durations (Fig. 1G) with HF-*S. pneumoniae*, as indicated. Subsequent quantitation of surviving IB4-positive cells did not show a significant reduction of relative microglial viability after incubation with $10^5$–$10^9$ HF-*S. pneumoniae/ml during the whole round of observation. In contrast, incubation with $10^8$ HF-GBS/ml led to a significant reduction of relative microglial viability after 72 h, as outlined above.

In summary, HF-GBS induces apoptosis in microglia in a dose- and time-dependent manner.

**Microglial apoptosis induced by HF-GBS is not dependent on LTA**

LTA is a conserved amphiphilic constituent of the cell wall in Gram-positive bacteria (37). It shares some features with the potent toxin LPS derived from Gram-negative bacteria, and has thus been proposed as being the functional equivalent in Gram-positive organisms (38). To assess the role of LTA in GBS-induced microglial apoptosis, microglia were purified from cortices of wild-type mice and subsequently incubated with various concentrations of GBS LTA for 72 h. Cells were then stained with IB4 and quantified (Fig. 2A). In contrast to the effect of whole GBS, GBS LTA did not significantly reduce microglial viability, even when used at very high concentrations. This finding was confirmed with LTA from *S. aureus*, which exceeds GBS LTA in inflammatory potency (24). *S. aureus* LTA did not induce cell death in microglia, even when used in concentrations 100-fold above those required to induce the release of cytokines (38). To extend these studies, microglial cells were incubated with HF-GBS COH1, NEM 316, or HF-*S. pneumoniae*, which represents the primary cause of bacterial meningitis in children after the age of 3 mo, was analyzed for cytotoxic properties in microglial cultures. To this end, purified wild-type microglia were incubated at various doses (Fig. 1F) for various durations (Fig. 1G) with HF-*S. pneumoniae*, as indicated. Subsequent quantitation of surviving IB4-positive cells did not show a significant reduction of relative microglial viability after incubation with $10^5$–$10^9$ HF-*S. pneumoniae/ml during the whole round of observation. In contrast, incubation with $10^8$ HF-GBS/ml led to a significant reduction of relative microglial viability after 72 h, as outlined above.

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In summary, HF-GBS induces apoptosis in microglia in a dose- and time-dependent manner.
microglia were prepared from forebrains of both B6Smn.C3-diatated by FasL (44, 45). AICD in lymphocytes (41). Microglial cells express both Fas/FasL plays a crucial role in the regulation of apoptosis of microglial cell death. FasL is a type II transmembrane glycoprotein that binds to Fas, a transmembrane protein belonging to the TNFR family. After binding to FasL, Fas activates an intracellular signaling pathway that leads to apoptosis of the target cell (40). Fas/FasL plays a crucial role in the regulation of apoptosis of AICD in lymphocytes (41). Microglial cells express both Fas and FasL (42, 43) and are highly susceptible to apoptosis mediated by FasL (44, 45).

To assess the role of FasL in GBS-induced microglial cell death, microglia were prepared from forebrains of both B6Smn.C3-Fasl−/− (gld) mice lacking FasL and C57BL/6J wild-type mice. Cultures of microglia were incubated with various doses of HF-GBS for 72 h (Fig. 3A) or with 10^8 HF-GBS/ml for various incubation times (Fig. 3B), as indicated. Subsequently, IB4-positive cells were quantified. HF-GBS induced a significant reduction of IB4-positive cells in both wild-type cultures and cultures derived from gld mice, in a dose- and time-dependent fashion. Despite the fine titration of HF-GBS, we did not observe a significant difference between wild-type and gld microglia with respect to viability after incubation with HF-GBS. As a positive control for FasL-induced cell death, purified microglia derived from C57BL/6J mice were incubated with increasing concentrations of FasL for 72 h (Fig. 3C). Subsequently, IB4-positive cells were counted, and results were statistically expressed as relative microglial viability. Dose-response experiments revealed a significant reduction in relative microglial viability after incubation with 0.1 ng/ml FasL.

In summary, these results demonstrate that FasL is not fundamentally involved in HF-GBS-induced microglial cell death.

**Microglial apoptosis induced by HF-GBS is not due to soluble factors, such as NO**

NO is a key mediator of GBS- and pneumococcus-induced apoptosis in macrophages (46, 47) and plays a major role in GBS-induced neuronal injury via microglia. To determine the role of soluble molecules secreted by activated microglia in HF-GBS-induced microglial apoptosis, microglial cells from wild-type mice were incubated either with 10^8 HF-GBS/ml or PBS (control) for 72 h. Supernatants were then filtered through a 0.2-μm filter to both remove bacteria and save soluble molecules. Subsequently, freshly prepared wild-type microglia were incubated with the harvested supernatants. Microglia incubated with 10^8 HF-GBS/ml served as a positive control. Cell cultures were then stained with IB4 and analyzed by TUNEL assay (Fig. 4A). Whereas whole HF-GBS induced TUNEL positivity in most IB4-positive cells after 36 h, no TUNEL staining was observed in cultures treated with supernatants from HF-GBS-stimulated microglia for 36 h (data not shown) and 72 h (Fig. 4A). Data for the positive controls are shown for the 36-h time point because only few microglia were still viable after 72 h. To ensure that these results were not due to extended incubation times or labile cell-toxic molecules, the above experiment was repeated with supernatants harvested after 12, 24, 36, and 48 h of HF-GBS treatment. In concordance with the results described above, no TUNEL-positive cells were found (data not shown).

To confirm that soluble molecules are not responsible for GBS-induced microglial apoptosis, we conducted experiments using Transwell chambers. Microglia were placed in the lower chamber,
and the upper chamber inserts contained microglia or no cells. Next, $10^8$ HF-GBS/ml was added to the upper chamber for 12, 24, 36, 48, or 72 h. The addition of $10^8$ HF-GBS/ml for 36 h to the lower chamber served as a positive control. Cell cultures of the lower chamber were then stained with IB4 and analyzed by TUNEL assay (Fig. 4B). Whereas HF-GBS-induced distinct TUNEL positivity in cultures treated with HF-GBS in the lower chamber, as observed above, only sporadic TUNEL staining was observed in the lower chamber when cultures in the upper chamber were treated with HF-GBS for various times, as indicated. Under these circumstances, the percentage of TUNEL-positive cells in the lower chamber did not significantly differ from control cultures (time 0 h).

To examine whether NO contributes to GBS-induced microglial cell death, wild-type microglia were incubated with either the inducible NO synthase inhibitor aminoguanidine (200 μM) or vehicle (control). After 2 h, HF-GBS was added, and after further 72 h of incubation, IB4-positive cells were quantified (Fig. 4C). In accordance with the data presented above, HF-GBS induced a significant reduction of IB4-positive cells without a measurable effect of aminoguanidine on this process. Notably, aminoguanidine abrogated HF-GBS-induced formation of NO throughout the entire period of observation (Fig. 4D).

In summary, these results indicate that HF-GBS-induced apoptosis in microglia is not caused by secreted factors and in particular not by NO.

**Microglial apoptosis induced by HF-GBS and live GBS is dependent on MyD88 and TLR2**

To test whether GBS-induced toxicity in microglia requires functional MyD88 and TLR2, purified microglial cells from C57BL/6J, MyD88$^{-/-}$, and TLR2$^{-/-}$ mice were treated with $10^8$ HF-GBS/ml for 36 h, and apoptosis was assessed by TUNEL assay (Fig. 5A). Whereas we routinely observed positive TUNEL staining in $\sim$95% of wild-type microglial cells, hardly any TUNEL positivity was detected in microglia derived from MyD88$^{-/-}$ and TLR2$^{-/-}$ mice (<2% of cells) (Fig. 5B). To confirm that TLR2 and MyD88 are crucial for GBS-induced toxicity, we determined relative microglial viability after treatment with HF-GBS for 72 h. In accordance with the data presented above, microglia derived from MyD88$^{-/-}$ and TLR2$^{-/-}$ mice were completely protected against HF-GBS-induced cell death (Fig. 5C).

The described experiments using HF bacteria were intended to reveal interactions between the GBS cell wall and microglia. It seemed necessary to determine whether the interaction of GBS with TLR2 had an impact on microglial viability in the context of an in vivo infection model in which live GBS replicates and actively secretes molecules. Thus, purified microglial cells derived from C57BL/6J or TLR2$^{-/-}$ mice were incubated with $10^6$ (Fig. 6A) and $10^8$ (Fig. 6B) CFU live GBS/ml for various times, as indicated. Subsequently, cells were quantified, and relative microglial viability was assessed, setting the viability of control cells to 100%. We observed a significant decrease in microglial viability as early as 1.5 h after addition of live GBS. In contrast, microglia derived from TLR2$^{-/-}$ mice did not show decreased viability throughout the entire observation. Notably, live GBS induced cell death in microglia at a much faster rate than HF-GBS. However, in both cases, cell death was largely dependent on TLR2. Thus, nonspecific toxicity by GBS growing in culture appears to be negligible in our model.

However, soluble molecules such as H$_2$O$_2$ might account for the differences in cell-death kinetics between microglia stimulated...
with live or HF-GBS. Live GBS produces and releases H₂O₂, which has been shown to mediate apoptosis in microglia in response to S. pneumoniae (48, 49). To assess the role of H₂O₂ in GBS-induced microglial apoptosis, H₂O₂ formation by live GBS was inhibited by the addition of catalase 1.5 h before exposure to wild-type microglia. The time course (1.5–9 h) and dose dependence (10⁶, 10⁷, and 10⁸ CFU live GBS/ml) of microglial viability did not reveal differences between catalase-treated or nontreated microglia (data not shown). Thus, H₂O₂ appears not to be involved in microglial apoptosis induced by GBS.

It is well established that glucose can protect the immature brain from neuronal damage under specific circumstances (50, 51). Furthermore, we observed that the content of glucose decreased rapidly in both medium from wild-type and TLR2-deficient microglia during incubation with live GBS (S. Lehnardt, unpublished data). To exclude the possibility that glucose consumption by proliferating GBS and ensuing low glucose concentration in the medium caused microglial cell death, we repeated the experiments described above with the distinction that culture medium was substituted with fresh glucose (constant concentration at 4.5 mg/ml) during the whole length of the experiment. Glucose substitution remained without effect on microglial viability (data not shown).

FIGURE 5. GBS-induced microglial cell death requires MyD88 and TLR2. Microglial cells derived from C57BL/6J, MyD88⁻/⁻, or TLR2⁻/⁻ mice were incubated with 10⁸ HF-GBS/ml for 36 h. Cells were then stained with IB4, fixed, and analyzed by TUNEL assay (A). Scale bar, 50 μm. Quantitation of TUNEL-positive microglia in the presence or absence of 10⁸ HF-GBS/ml (B). Microglial cells prepared from C57BL/6J, MyD88⁻/⁻, and TLR2⁻/⁻ mice were incubated with 10⁸ HF-GBS/ml for 72 h. Surviving IB4-positive cells were quantified, and results were statistically expressed as relative microglial viability (C). Similar results were obtained in three experiments. The results are presented as mean ± SD. p < 0.001 (Student’s t test).

Thus, the content of glucose in the medium does not play a major role in microglial cell death induced by live GBS in vitro.

In summary, microglial cell death induced by HF-GBS and live GBS requires a functional MyD88 and TLR2 pathway in microglia. No qualitative difference was observed between HF-GBS and live GBS regarding dependence on TLR2.

HF-GBS-induced microglial cell death requires activation of caspase-8 that is dependent on TLR2, but does not involve caspase-3

The caspase family of cysteine-directed proteases represents one of the key regulators of apoptosis. Caspase-3 is a terminal effector molecule that, once activated, commits cells to death. GBS-induced apoptosis involves the activation of caspase-3 in macrophages (52). In this study, we assessed the role of caspase-3 in GBS-induced microglial apoptosis. Accordingly, lysates from microglia incubated with HF-GBS were tested for caspase-3 activation by Western blot with an Ab directed against cleaved caspase-3 (Fig. 7A). No cleaved caspase-3 was detected in HF-GBS-treated microglia, whereas this fragment was detected in microglia treated for 12 h with staurosporine. To further analyze the role of caspase-3 in GBS-induced microglial apoptosis, wild-type microglia were incubated either with the caspase-3-specific inhibitor DEVD-CHO or the broad-spectrum caspase inhibitor Z-VAD-FMK 2 h before treatment with HF-GBS. After 36 h, cells were stained with IB4 and TUNEL assay and were quantified (Fig. 7B). Whereas general inhibition of caspases significantly reduced HF-GBS-induced TUNEL positivity in microglia, specific inhibition of caspase-3 did not reduce the number of TUNEL-positive cells.
after incubation with HF-GBS. Microglial cells incubated with DEVD-CHO or Z-VAD-FMK alone were TUNEL-positive to a similar extent as it was observed for cells under control conditions. Thus, whereas activation of caspases in general appeared to be critical for HF-GBS-induced cell death in microglia, the activation of caspase-3 was dispensable in this context.

Initiator caspases, such as caspase-8, are upstream caspases in apoptotic pathways and are triggered by the engagement of death receptors (53). TLR2-mediated apoptosis in HEK293 cells is executed by Fas-associated death domain protein and caspase-8 (25). To investigate the role of caspase-8 in GBS-induced apoptosis, wild-type and TLR2−/− microglia were incubated with 10⁸ HF-GBS/ml for 24 h. Cells were then live stained with Image-iT LIVE Green Caspase-8 dye (C). Scale bar, 50 μm. Wild-type microglia were treated with either Z-VAD-FMK or Z-IETD-FMK for 2 h before the incubation with 10⁸ HF-GBS/ml for 36 h. Cells were then stained with IB4 and TUNEL assay and quantified. Experiments are presented as mean ± SD. p < 0.001 (Student’s t test) for the comparison of the indicated groups with control and GBS (D). Microglia were incubated with 10⁸ HF-GBS/ml in the presence of Z-IETD-FMK for 24 h and subsequently analyzed by the Image-iT LIVE Green Caspase-8 Detection assay. Scale bar, 10 μm (E). All experiments were performed three times.

Next, we complemented our loss-of-function study on the role of TLR2 and caspase-8 with a gain-of-function model. Human hepatoma cells (HuH7) were transfected with TLR2 and a reporter that encoded for the promoter element of caspase-8 (bp −470/−76). Transcriptional activation of caspase-8 was induced by HF-GBS in a dose-dependent fashion, and TLR2 was essential for this activation (Fig. 8A). In contrast to the effects observed with GBS, LTA did not induce the caspase-8 reporter (data not shown). Notably, HuH7 cells expressed a low amount of endogenous TLR2, as determined by RT-PCR (data not shown), which might account for background stimulation in these cells. However, heterologous expression of TLR2 consistently increased this response. Furthermore, transcriptional activation of caspase-8 by HF-GBS was dependent on the Ets family of transcription factors, because a −470/+76 caspase-8 reporter containing a point mutation in an Ets-like element binding site was not activated by GBS (Fig. 8B).

In summary, HF-GBS-induced activation of caspase-8 is dependent on a functional TLR2 and the Ets family of transcription factors.
of microglia to GBS. Fourth, apoptosis in microglia is a direct consequence of caspase-8 activation and does not involve feedback loops comprising soluble toxins as reported for microglial-induced neuronal apoptosis.

In this study, we attempted to define whether LTA was involved in the interaction between microglia and GBS. LTA was an attractive candidate in this context because it is a GBS-derived ligand of TLR2 and a well-established TLR2 ligand of Gram-positive bacteria in general (24). However, several lines of evidence strongly suggest that LTA is of minor, if any, significance in GBS-induced cell death of microglia. First, although we found purified LTA to be capable of cell activation, we were unable to induce apoptosis in microglia with the same preparation, even when used in excessive concentrations (58). Second, a mutant GBS type that expressed deanylminated LTA induced apoptosis in microglia to a similar extent as in the isogenic parental strain. This finding has important implications. GBS must contain a pathogen-associated molecular pattern other than LTA that induces apoptosis via the engagement of TLR2. Moreover, stimulation of TLR2 alone is not sufficient to drive programmed cell death in microglia. This observation corresponds to a recent study by Jung et al. (57), who failed to induce microglial apoptosis with purified TLR2 agonists. It is tempting to speculate that GBS comprises a second, TLR2-independent molecule that synergizes with the TLR2 agonist for the induction of apoptosis.

Downstream of TLR2, we identified MyD88 and caspase-8 as crucial factors for GBS-induced apoptosis in microglia. Caspase-8 underlies significant posttranslational modification. The coupling of Fas-associated death domain protein results in the proteolytic cleavage of procaspase-8 into p18 and p10. These subunits are then released to the cytosol and mediate caspase activity via a C-terminal protease domain (59). In addition to posttranslational modifications, transcriptional regulation of caspase-8 appears to be essential for apoptosis, because heterologous expression of caspase-8 is sufficient to induce cell death (60). Expression of caspase-8 is reduced in a number of tumors such as neuroblastomas and neuroectodermal brain tumors due to somatic mutations and hypermethylation of genomic caspase-8 sequences (61, 62). Accordingly, we assessed in a gain-of-function approach whether GBS induces transcription of caspase-8 in vitro. We found that GBS induces caspase-8, and TLR2 and the Ets family of transcription factors are critical in this context. MAPKs can be assumed to be involved in caspase-8 activation because they have previously been shown to be essential for the activation of the Ets family of transcription factors that comprises ternary complex factors, such as Elk1 and Sap1a/b (63).

Besides TLRs, other molecules expressed by the host cell, such as FasL, have been implicated in apoptosis. It is well established that Fas/FasL play a crucial role in the regulation of apoptosis of AICD in lymphocytes (41). Microglia are particularly susceptible to FasL-induced apoptosis (44, 45). Using FasL-deficient mice, we showed that GBS-induced cell death is not dependent on the expression of FasL. This result is in accordance with a previous finding that a neutralizing Ab against FasL does not prevent LPS-induced apoptosis in the microglial cell line BV-2 (19). Mechanisms leading to AICD independently of Fas-FasL interaction are known: AICD in thymocytes does not involve FasL, but rather requires TRAIL or the activation of Bim, a member of the Bcl-2 family (64, 65). It remains to be elucidated whether molecules such as TRAIL are also involved in GBS-induced apoptosis of microglia in a manner similar to their role in GBS-induced apoptosis in monocyes (55).

Previous studies have identified microglial NO as a key mediator of GBS-induced neuronal injury and LPS-induced microglial apoptosis (12, 66). In contrast, we report in this study that NO or

![Figure 8](http://www.jimmunol.org/ji/6141_SI/6141_SI_f8.jpg)
other secreted soluble molecules are not required for GBS-induced apoptosis in microglia.

Against the background of the data discussed above, the question arises how GBS-induced microglial cell death relates to the observation that gliosis follows various forms of CNS injury in vivo. Gliosis comprises several cell types, including reactive astrocytes, microglia, and oligodendrocytes. The relative contribution of the different glial subpopulations to gliosis varies greatly depending on the disease condition (67). Because the data presented in this study on microglia were raised in vitro, our model does not necessarily reflect all aspects leading to gliosis under the complex inflammatory conditions found in vivo. However, our finding that the interaction between GBS and TLR2 results in microglial cell death may suggest that the CNS has evolved mechanisms to limit the induction of gliosis in the context of GBS-induced infections. Ultimately, the role of GBS-induced microglial cell death needs to be further elucidated in in vivo models.

It has been a long-standing dispute whether bacteria-induced apoptosis of phagocytes is advantageous or harmful for the host. On the one hand, this process leads to the clearance of internalized bacteria, and therefore limits inflammation. In contrast, apoptosis of phagocytes is detrimental to the host because it may weaken the first-line immune defense (68). We speculate that apoptosis of phagocytes is per se neither good nor bad, but can be advantageous or disadvantageous for the fate of the host depending on infection-specific conditions, such as the causative bacterial species or the bacterial load. In line with this, both TNF-α and the TNF-inducing signaling intermediate MyD88 lower lethality in a low-dose (LD10) model of neonatal GBS sepsis in mice, but increase lethal days in a high dose (LD90) model of neonatal GBS meningitis (20, 66). On the basis of our recently, in astrocytes and microglia (20, 66). On the basis of our finding that the interaction between GBS and TLR2 results in microglial apoptosis via a signaling cascade, which triggers cell death in microglia via a signaling cascade, which shares molecules with the pathway that instructs the GBS-induced inflammatory host response in the CNS. Accordingly, TLR2 and MyD88 may be regarded as elaborate switches that mediate the self-control of CNS inflammation. Thus, it remains an important goal to decipher whether insufficient AICD in newborn infants contributes to neuronal damage in GBS meningitis to develop new therapeutic strategies.

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Disclosures
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References

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