Lipoproteins of Listeria monocytogenes Are Critical for Virulence and TLR2-Mediated Immune Activation

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Lipoproteins of *Listeria monocytogenes* Are Critical for Virulence and TLR2-Mediated Immune Activation\(^1\)

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Numerous cell surface components of *Listeria* influence and regulate innate immune recognition and virulence. Here, we demonstrate that lipidation of prelipoproteins in *Listeria monocytogenes* is required to promote NF-κB activation via TLR2. In HeLa cells transiently expressing TLR2, *L. monocytogenes* and *Listeria innocua* mutants lacking the prolipoprotein diacylglyceryl transferase (*lgt*) gene are unable to induce TLR2-dependent activation of NF-κB, a property intrinsic to their isogenic parental strains. TLR2-dependent immune recognition is directed to secreted, soluble lipoproteins as evidenced by the sensitivity of the response to lipoprotein lipase. Studies of bone marrow-derived macrophages of C57BL/6 wild-type and TLR2-deficient mice infected with wild-type and *lgt* mutant strains indicate that the absence of host TLR2 receptor signaling has consequences similar to those of the absence of the bacterial TLR2 ligand, i.e., a delay in cellular immune responses directed toward the bacterium. Infection studies with the wild-type and TLR2\(^{-/-}\) mice indicated attenuation of the *lgt* deletion mutant in both mouse strains, implying multiple roles of lipoproteins during infection. Further characterization of the Δ*lgt* mutant indicated that it is impaired for both invasion and intracellular survival and exhibits increased susceptibility to cationic peptides. Our studies identify lipoproteins as the immunologically active ligand of TLR2 and assign a critical role for this receptor in the recognition of these bacteria during infection, but they also reveal the overall importance of the lipoproteins for the pathogenicity of *Listeria*. *The Journal of Immunology*, 2008, 181: 2028–2035.

The innate immune system provides a rapid response to pathogens through primary recognition of bacterial components, known as pathogen-associated molecular patterns (PAMP),\(^2\) via ligation of signaling receptors in the host. The TLRs have been identified as major players in the early detection of microbes by activating signal cascades and mediating the induction of NF-κB and IFN regulatory factor 3, leading to immune activation and release of proinflammatory cytokines. The TLR family members are transmembrane or intraendosomal proteins consisting of extracellular N-terminal leucine-rich repeat motifs, followed by a cysteine-rich region, a transmembrane domain, and an intracellular Toll/IL-1R motif. More than 10 members of the human TLR family have been identified, having diverse yet predetermined ligand specificity such as stimulation by viral RNA and detection of bacterial components. Of particular interest to the study of bacterial pathogenesis are TLR4, which recognizes LPS, TLR5, which senses bacterial flagellin, and TLR2, which was reported to detect a number of different PAMPs, including lipoteichoic acid, peptidoglycan, and lipoproteins (1–3).

*Listeria monocytogenes* is a Gram-positive facultative intracellular bacterium and the causative agent of listeriosis, a serious disease with clinical symptoms such as septicemia and meningitis that primarily affects immunocompromised hosts, including newborns, transplant or cancer patients, and the elderly. The bacterium enters the host via ingestion of contaminated food and crosses the intestinal barrier where it can trigger innate immune mechanisms. *L. monocytogenes* exhibits multiple TLR ligands (4), and the importance of TLR2 recognition during infection with *L. monocytogenes* has been described previously (5). In vivo studies with TLR2-deficient mice demonstrated increased bacterial loads and reduced activation of macrophages (5), indicating a principal role for TLR2 in controlling *Listeria* infection.

Current studies have highlighted the role of lipoproteins as the dominant immune activation factor of Gram-positive bacteria (6, 7). Lipoproteins are membrane-associated proteins containing a consensus sequence at the C-terminal end of the signal peptide, referred to as a lipobox. It is composed of the amino acids leucine—alanine/serine—glycine—glycine—proline followed by a requisite conserved cysteine. The thiol group of the cysteine is lipid modified by introducing a thrombril linkage to a diacylglycerol moiety (8), a reaction conducted by prolipoprotein diacylglycerol transferase, and the resulting prolipoprotein is further processed by the lipoprotein-specific signal peptidase (9). After cleavage of the signal peptide, the conserved cysteine residue becomes the N terminus of the mature lipoprotein and the newly established N-terminal amino group is acetylated with a fatty acid residue (8). Recently, a deletion mutant of *L. monocytogenes* lacking the prolipoprotein diacylglycerol transferase gene (*lgt*) was characterized (10) that is unable to transfer the diacylglycerol moiety from phosphatidylglycerol to generate the modified prolipoproteins. As a consequence,
membrane retention of unmodified lipoprotein precursors is lost. Baumgärtner et al. showed that translocation, however, is not affected given that signal peptide removal can still be performed by lipoprotein-specific signal peptidase II (10).

Related studies have demonstrated that lipoproteins are essential for the virulence of Streptococcus and Staphylococcus species (6, 7, 11, 12). For Streptococcus, it was shown that lgt is not essential for cell growth in vitro but is essential for viability during infection. Similarly, a transposon-induced lgt mutant of Staphylococcus aureus elicited only decreased immune response in host cells after infection, due to the absence of mature lipoproteins.

In this work, we used an lgt deletion strain of L. monocytogenes EGD-e (10) to study the role of lipoproteins for survival within the host and to examine the impact of lipoproteins on TLR2 recognition of Listeria. To determine whether TLR2 recognition is specific for the pathogenic species or if it is a general characteristic of the genus Listeria, we also constructed an L. innocua lgt deletion strain and examined its ability to induce TLR2 mediated NF-κB activation. We were able to demonstrate the requirement of bacterial lipoproteins for the TLR2-dependent induction of the stress signal transcription factor NF-κB and the early secretion of inflammatory cytokines during infection with Listeria. Lack of functional lipoproteins, however, results in diminished virulence of L. monocytogenes and decreased survival in the animal model of infection.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice were purchased from Harlan Winkelmann and used for in vivo infection experiments. Female TLR2−/− mice on a C57BL/6 background were donated by M. Steinmueller (Medical Clinic II, Justus-Liebig-University, Giessen, Germany). All animals were kept under controlled pathogen-free conditions. All work conducted in this study is covered by license GI15/5-26/2004 and approved by the regional clinic II, Justus-Liebig-University, Giessen, Germany). All animals were kept under controlled pathogen-free conditions. All work conducted in this study is covered by license GI15/5-26/2004 and approved by the regional clinic II, Justus-Liebig-University, Giessen, Germany). All animals were kept under controlled pathogen-free conditions. All work conducted in this study is covered by license GI15/5-26/2004 and approved by the regional clinic II, Justus-Liebig-University, Giessen, Germany). All animals were kept under controlled pathogen-free conditions. All work conducted in this study is covered by license GI15/5-26/2004 and approved by the regional clinic II, Justus-Liebig-University, Giessen, Germany).

Bacterial strains

L. monocytogenes EGD-e and L. innocua, as well as their isogenic mutants, were grown in brain-heart infusion (BHI) broth or on BHI agar plates (Difco) at 37°C. Escherichia coli was grown in Luria-Bertani broth at 37°C. When appropriate, antibiotics were added to the following concentrations: erythromycin, 300 μg/ml for E. coli and 5 μg/ml for Listeria; chloramphenicol, 25 μg/ml for E. coli and 8 μg/ml on agar plates or 5 μg/ml in broth for Listeria; 0.5 μg/ml gallerimycin, 32 μg/ml colistin, and 20 μg/ml polymyxin B for infection assays. Bacteria were grown in BHI and harvested at exponential phase (OD600~1.0), and the bacterial concentration was adjusted by OD.

Deletion of the lgt gene in the genome of L. innocua was done as described (13). Briefly, the flanking regions of the lgt gene were amplified by the PCR (Pfu polymerase) using the primer pairs LIRlgtFor1 5′-TTATTTGAGCTGCAGTGTTGCTTTC-3′ and LIRlgtRev2 5′-AAAAATTCACATTTAATCCCCATCTTTAAAAAAGAAC3′, giving rise to a 403-bp product I, and LIRlgtFor3 5′-GGAATGAGGGAAATTAAGGTTGAGGATGTTGTA-3′ and LIRlgtRev4 5′-CTCGCTGTCGACACTCCATCATACTC-3′, generating a 337-bp product II. A second PCR was performed with primers LIRlgtFor1 and LIRlgtRev4 and using both products I and II as template to amplify a 711-bp DNA fragment, which was subsequently digested (SacI and SalI, underlined), ligated into the temperature-sensitive E. coli/Listeria shuttle vector pVALA (14) and transformed into E. coli. The vector construct was isolated, sequenced and electroporated into L. innocua to achieve gene deletion.

For generating the complemented L. monocytogenes Δlgt strain, the lgt gene was amplified by the PCR using primers 2482F-P1gt 5′-ATTGGGATCTGATAAGCA-3′ and 2482R-lgt 5′-CTTAACTCAAATCGGAAACTT-3′ and L. monocytogenes EGD-e genomic DNA as template. The 1185-bp product was cloned into the integration vector pPL2 (15) using the restriction sites BamHI and XhoI (underlined). The construct was confirmed by sequencing and then transformed into the L. monocytogenes Δlgt deletion strain to obtain L. monocytogenes Δlgt atBΔlgt, also referred to as Δlgt-lgt. Strains used in this study are listed in Table 1.

Isolation of supernatant proteins

Bacterial cells were grown to exponential phase in BHI and centrifuged at 5000 × g for 15 min at 4°C, after which culture supernatant was collected. Proteins were precipitated with 10% TCA, resuspended in 1 M Tris-HCl (pH 8.8) and stored at −20°C. Samples were separated by SDS-PAGE (12.5%), and proteins were visualized by staining with Coomassie Brilliant Blue G-250.

Luciferase reporter assay

For determining NF-κB activation, the luciferase reporter assay was performed with HeLa cells transiently expressing human TLR2 as well as the reporter plasmids pELAM-Luc and plрHL-TK. The human TLR2 expression plasmid pTL2 was kindly provided by C. Kirchning (Institute of Medical Microbiology, Immunology and Hygiene, TU, Munich, Germany). The plasmid pELAM-Luc, expressing luciferase under the control of the NF-κB binding E-selectin promoter, was kindly provided by J. Chow (Eisai Research Institute, Andover, MA) (16). The vector constitutively expressing Renilla luciferase, pRL-TK (Promega), was used for signal normalization. The background signal was determined using the empty CMV promoter vector pRK5 (BD Pharmingen).

Transient overnight cultures of HeLa cells in six-well plates, grown in DMEM (Life Technologies) supplemented with 10% FCS (PAA Laboratories) and 1× penicillin-streptomycin (Invitrogen), were washed in DMEM and transfected with a mix of 4.8 μg of plasmid DNA and 15.5 μl of Lipofectamine 2000 (Invitrogen). After 5 h, medium was replaced with DMEM supplemented with 10% FCS; after an additional 2 h, the cells were split, transferred to 96-well plates at 4 × 103/well, and incubated overnight in a humidified atmosphere (5% CO2). Listeria was plated to an OD of 0.1 to obtain a suspension of 3 × 107 CFU/ml, harvested by centrifugation, and resuspended in DMEM containing 1% FCS; 20 μl aliquots were added to each well for a multiplicity of infection (MOI) of 15. For stimulation with culture supernatants, bacteria were grown at 37°C in BHI for 12 h. Clear supernatant was collected by centrifugation at 5000 × g for 10 min, and 20-μl aliquots were added to each well. Control samples were treated with an equal volume of sterile BHI medium. For treatment with pneumococcal lipoprotein lipase (Sigma-Aldrich), 100-μl supernatant samples were incubated with increasing amounts of enzyme for 30 min at 37°C, followed by inactivation at 72°C for 20 min, and 20-μl aliquots were added to the transfected HeLa cells. Stimulated cells were incubated for 5 h in DMEM supplemented with 1% FCS, washed with PBS, lysed with 20 μl of passive lysis buffer (Promega) and stored at −20°C. Measurement of NF-κB-mediated luciferase activity was conducted with the Dual-Luciferase Reporter Assay (Promega) using an L-max plate reader (Molecular Devices). Signal measurements were performed in triplicate, and experiments were repeated at least three times. The primary firefly luciferase signal was normalized to the signal of Renilla luciferase in each well, resulting in relative luciferase activity. Results are presented as fold changes of stimulated activity to nonstimulated activity.

Cytokine assays

Bone marrow-derived macrophages were isolated from 4- to 6-week-old C57BL/6 wild-type and TLR2-deficient mice and grown and differentiated for 7 days in L929 conditioned medium to an approximate concentration of 5 × 106 cells/well in 24-well plates. Supernatants were collected from cells stimulated for 4, 8, and 12 h with L. monocytogenes, L. innocua, or their isogenic Δlgt mutants in DMEM containing 50 μg/ml gentamicin. The expression levels of cytokines TNF-α and IL-6 were measured on a Biotek spectrophotometer (Bio-Rad) using the appropriate X-plex mouse cytokine assay (Bio-Rad) following the manufacturer’s instructions.

Virulence studies

HeLa or Caco-2 cells, cultured in 24-well plates in MEM (Life Technologies) supplemented with 10% FCS, were infected with 1 × 106 bacteria to obtain an MOI of 10. P388D1 macrophages, cultured in 24-well plates in RPMI (Life Technologies) supplemented with 10% FCS, were infected with 1 × 105 bacteria to obtain an MOI of 10 and 5 × 105 bacteria to obtain an MOI of 50. At 1 h postinfection, medium was removed, and cells were washed twice with PBS and incubated for 1 h in cell culture medium containing 50 μg/ml gentamicin. Cells were then washed three times with PBS and lysed with ice-cold 0.2% Triton X-100, and the released bacteria were plated on BHI agar plates in appropriate dilutions were quantified after overnight incubation at 37°C. L.292 cells were cultured in six-well plates in RPMI supplemented with 10% FCS, infected with a similar MOI and then overlaid with 0.7% agarose in DMEM containing 10% FCS and 10 μg/ml gentamicin. After 4 days of incubation at 37°C in 5% CO2, plaques were visualized with 0.3% neutral red in PBS.

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nonpathogenic strains such as *L. innocua*, we created an *lgt* deletion mutant of *L. innocua* (LIR Δ*lgt*; see Table I) using standard homologous recombination techniques. *L. innocua* is avirulent and incapable of invading epithelial cells, such as the HeLa cell line, or to grow in macrophages. Thus, the strain is suitable for assessing immune responses that are independent of virulence properties. As detailed below, both Δ*lgt* strains, *L. monocytogenes* and *L. innocua*, were examined for their ability to induce a NF-κB-based luciferase reporter after in vitro infection of hTLR2-expressing HeLa cells, as well as for their ability to induce cytokine release in bone marrow-derived mouse macrophages.

**Lack of diacylglycerol-modified lipoproteins abolishes TLR2-mediated NF-κB activation**

TLR2-dependent recognition of *L. monocytogenes*, *L. innocua*, and their isogenic *lgt* deletion mutants was investigated by monitoring NF-κB activation in cells coincubated with these bacteria using the Dual-Luciferase Reporter Assay System. Cell stimulation was performed with either bacterial cells or culture supernatants on transfected HeLa cells transiently expressing the firefly luciferase gene under the control of an NF-κB-dependent promoter. Cotransfection of a plasmid constitutively expressing *Renilla* luciferase was used for normalization of luciferase activity. To assess TLR2-dependent activity, cells were additionally transfected with either a CMV promoter-driven plasmid expressing human TLR2 or an empty CMV promoter vector. Relative activity of firefly to *Renilla* luciferase was calculated, and the fold change of the normalized signal for stimulated vs nonstimulated cells was determined (Fig. 2). In coinoculation experiments with either viable bacteria or culture supernatants, TLR2-mediated NF-κB-dependent luciferase activity is induced 5-fold when comparing wild-type with the Δ*lgt* mutant bacteria (Fig. 2, A and B). This effect is specific for the TLR2-expressing cells, given that no differences between the wild-type and mutant strains are apparent in cells bearing the empty vector. The NF-κB-dependent luciferase activation by culture supernatants in the absence of bacterial cells demonstrates the role of soluble lipoproteins as PAMPs recognized by the extracellular TLR2 receptors. Also, TLR2 recognition is sufficient for NF-κB activation regardless of bacterial virulence or adhesion properties. Furthermore, treating culture supernatants derived from *L. monocytogenes* EGD-e with lipoprotein lipase decreases the stimulatory signal in a dose-dependent manner (Fig. 2C). By reintroducing the *lgt* gene into the *L. monocytogenes* Δ*lgt* mutant, it is possible to restore induction levels of NF-κB-dependent luciferase activity to those of the wild-type EGD-e strain. Taken together, these data suggest that diacylglycerol-modified lipoproteins that are either secreted by the bacteria or bound to the bacterial surface are responsible for TLR2-mediated NF-κB activation.
TLR2 is required for early recognition of *L. monocytogenes*

Having demonstrated the involvement of lipoproteins in TLR2-mediated NF-κB activation, we next examined the role of lipoproteins in TLR2-dependent inflammatory responses. To this end, we examined cytokine induction in mouse bone marrow macrophages (BMM) derived from C57BL/6 wild-type and TLR2-deficient mice by coinoculation with the wild-type *L. monocytogenes* EGD-e and *L. innocua* strains or their respective Δlgt isogenic mutants. As expected, both parental strains activate BMM of C57BL/6 mice to induce the proinflammatory cytokines IL-6 and TNF-α (Fig. 3). In contrast, the levels of cytokine induced by the Δlgt deletion strains were strongly reduced at early times after infection (4 and 8 h). However, at 12 h postinfection, there was no difference in the levels of cytokines produced seen with the Δlgt deletion strains and their isogenic wild types. Reintroduction of the lgt gene by complementation of the *L. monocytogenes* Δlgt mutant restored cytokine levels to that seen with the wild-type EGD-e strain.

When BMMs derived from TLR2−/− mice were infected with either the wild-type strains or their isogenic Δlgt mutants, induction of the cytokines was virtually abolished at 4 and 8 h postinfection, but proinflammatory cytokine responses was observed for all strains at 12 h postinfection. Taken together, the results from these studies indicate that TLR2-mediated recognition is required for the early detection of *L. monocytogenes* during infection. Therefore, either absence of the TLR2 receptor on the host cell or that of the TLR2 ligand, i.e., lipoproteins, on the surface of bacteria has the same result, i.e., a delay in the immune responses mounted against these bacteria.

The Δlgt mutant is attenuated in vivo

The delayed cytokine response found in macrophages infected with the Δlgt mutant strain raised the question of the effects of lipoprotein deficiency on bacterial virulence and survival within the host. We infected C57BL/6 mice and their TLR2-deficient counterparts i.v. and quantified the bacterial loads in the liver and spleen at day 3 postinfection. The wild-type EGD-e strain showed higher colonization of both the spleens (3-fold) and livers (1.5-fold) of the TLR2-deficient mice as compared with organs of the wild-type mice (Fig. 4). We found that the Δlgt deletion mutant was highly attenuated in both the wild-type and TLR2 knockout animals and was recovered in numbers that were at least 100-fold less than that seen with EGD-e wild-type strain (Fig. 4).

Because the observed attenuation of the Δlgt mutant was independent of the TLR2 phenotype, we quantified bacterial loads of both the Δlgt mutant and the wild-type strain in the liver and spleen of BALB/c mice for 5 consecutive days postinfection. The study revealed that although the bacterial numbers of the parental mouse by coinoculation with the wild-type *L. monocytogenes* EGD-e and *L. innocua* strains or their respective Δlgt isogenic mutants.
L. monocytogenes EGD-e strain, at every single time point examined, significantly exceed that of the Δlgt mutant over the 5-day period examined, the kinetics of organ colonization by bacteria are similar for both strains (Fig. 5). Thus, it is the reduction in the numbers of mutant bacteria during early infection that is largely responsible for attenuation and suggests an intrinsic defect in the ability of these bacteria to either reach and/or to colonize target organs. We therefore examined the ability of these bacteria to survive in the peripheral blood of infected mice and to multiply in mouse serum preparations in vitro. The blood levels of wild-type bacteria were >30 times higher than that of the mutant strain after 6 h of infection with 380 vs 12 bacteria per ml (p = 0.0648). At 24 h postinfection, there was exuberant growth (>500-fold) of wild-type bacteria as compared with the Δlgt mutant (10,000 vs 17 bacteria per ml; p = 0.0540). No differences were observed in serum resistance between both bacteria (data not shown). The lowered numbers of viable mutant bacteria in peripheral blood after 24 h thus also account for the decreased numbers in the organs of infected mice.

The contribution of lipoproteins for listerial virulence was verified by infecting BALB/c mice with high lethal bacterial doses (2 × 10⁵ CFU), and survival of animals was followed for 10 days. As shown in Fig. 6, all mice infected with the Δlgt mutant survived over the 10-day period, whereas animals infected with the EGD-e parental strain died within 5 days.

The Δlgt mutation affects entry and survival in epithelial cells and macrophages

Apart from being poorly bacteremic, defects in the L. monocytogenes Δlgt mutant observed in the mouse infection model can also result from additional factors, including deficiencies in the bacteria for adhesion, internalization, escape from the phagolysosome, or cell-to-cell spreading. To narrow down these alternatives, we performed in vitro assays that allowed us to distinguish between the different stages of infection. We first examined the ability of the deletion mutant to invade non-phagocytic epithelial cell lines. The invasive capacity of the Δlgt mutant is strongly impaired with infection rates of only 20 or 10% that of the parental EGD-e strain in either HeLa or Caco-2 cell lines, respectively (Fig. 7A). Next, potential intracellular growth defects were assessed by infecting P388D1 murine macrophages with wild-type EGD-e or the Δlgt deletion mutant and then measuring intracellular bacterial counts at indicated time points postinfection. Whereas the uptake of the mutant is not affected, because higher infectious doses (MOI 50) resulted in higher intracellular bacterial numbers at 1 h postinfection, the growth of the deletion strain is greatly reduced compared with wild-type at later time points (Fig. 7B), independent of the initial bacterial loads. Finally, using a plaque-forming assay, which examines both intracellular growth and cell-to-cell spread, we found that the overall number of plaques formed by the Δlgt strain is lower than that of the wild-type strain, whereas the plaque size between the mutant and parental EGD-e strain differed by ~20% (p = 0.0216; Fig. 7C).

Lack of lipoproteins renders L. monocytogenes sensitive to cationic peptides

The important and varied roles of lipoproteins for the Gram-positive envelope have been discussed in detail by Sutcliffe and Russell (21). Because these functions can involve antibiotic resistance as well as substrate binding and transport, we wondered whether the lack of membrane-bound lipoproteins in the Δlgt deletion strain would also render L. monocytogenes sensitive to cationic antimicrobial peptides. Therefore, growth in the presence of gallidermin, polymyxin B, or colistin was evaluated for the parental EGD-e, the Δlgt mutant, and the complemented Δlgt-lgt strains (Fig. 8). Whereas the deletion mutant exhibits

![FIGURE 4. Control of in vivo infection by TLR2. C57BL/6 wild-type and TLR2-deficient mice were infected i.v. with EGD-e and Δlgt bacteria (2 × 10⁵ CFUs), and bacterial loads in spleen and liver were determined on day 3 postinfection.](http://www.jimmunol.org/)

![FIGURE 5. In vivo virulence in the mouse model of infection. The kinetics of bacterial growth was followed over 5 days in liver (A) and spleen (B) of BALB/c mice infected i.v. with EGD-e or Δlgt bacteria (2 × 10⁵ CFUs).](http://www.jimmunol.org/)
no growth defects when cultured in BHI only, in media supplemented with antimicrobials growth of the Δglt strain is reduced compared with the wild-type EGD-e strain. This deficiency can be alleviated by restoring the lgt gene, as evidenced by the growth characteristic of the complemented Δglt-lgt strain. This sensitivity to antimicrobials correlates with the decreased virulence of the bacteria as revealed in the in vitro and in vivo experiments outlined above (Fig. 4–7).

Discussion

**TLR2 detects Listeria early during infection by binding released lipoproteins**

Our data unequivocally demonstrate that lipoproteins of *L. monocytogenes* and *L. innocua* are the targets for recognition by TLR 2 and are independent of listerial virulence properties. Cell activation experiments with culture supernatant provide conclusive evidence that soluble components released into the environment are sufficient for microbial recognition by host cells expressing TLR2 and for induction of defense mechanisms by activation of NF-κB. Markedly, treatment of wild-type *L. monocytogenes* culture supernatant with lipoprotein lipase caused a decrease of stimulatory activity in a dose-dependent manner. It is likely that soluble lipopeptides are recognized before the host cell comes into direct contact with the bacteria. The significance of TLR2 in recognition of bacterial lipoproteins has also recently been reported for *S. aureus* (12, 22).

Our study showed an early induction (4 and 8 h postinfection) of the inflammatory cytokines TNF-α and IL-6 in macrophages infected with the *Listeria* parental strains but not with their isogenic Δglt mutants. In contrast, TLR2-deficient macrophages showed only weak induction at early time points (4 h, 8 h) with both wild-type and Δglt strains tested. At 12 h postinfection, we found high cytokine levels for both wild-type and Δglt-deficient bacteria regardless of the presence or absence of TLR2. The cytokine induction at later time points also suggests that recognition of PAMPs relies on redundant features and that signal triggering probably involves other extracellular receptors of the TLR family. Intracellular sensors of the TLR and Nod-like receptor family might also be involved in cytokine induction promoted by the Δglt mutants, given that both wild-type and mutant bacteria are phagocyted by macrophages. Possible additional candidates for signal-triggering include TLR5 and TLR9, responsible for recognition of flagellin and of non-methylated bacterial DNA respectively (18), or NOD1 and NOD2, receptors for catabolic products of peptidoglycan (23, 24). The idea of redundant-triggering is supported by studies with knockout mice that are deficient for the common protein adaptor of TLR signaling, myeloid differentiation factor 88 (MyD88) (5). As these mice are more susceptible to infection with *L. monocytogenes* than TLR2-deficient mice, this suggests TLR2 plays an important part in controlling infection but also that other MyD88-dependent signals are required for host resistance.

**FIGURE 7.** In vitro virulence studies with *L. monocytogenes* EGD-e and Δglt. Cells were infected either with the EGD-e or Δglt and lysed at indicated times; intracellular bacteria were determined in serial dilutions on BHI plates. A, Invasive properties of the EGD-e or mutant Δglt strains monitored 2 h after infection. B, Growth of intracellular bacteria in P388D1 murine macrophages. C, Plaque-forming assay of *L. monocytogenes* EGD-e and its isogenic deletion mutant Δglt. Monolayers of L929 cells were infected for 4 h and incubated for 4 days at 37°C, and plaques were visualized with neutral red. The Δglt mutant exhibits both lower plaque number and smaller plaque size (83%) than the wild-type strain.

**FIGURE 8.** Susceptibility to cationic peptides. Exponentially growing *L. monocytogenes* EGD-e, Δglt, and Δglt-lgt strains were treated with 32 µg/ml colistin (A), 0.5 µg/ml gallidermin (B), or 20 µg/ml polymyxin (C). OD (OD600) of growing bacteria was recorded as indicated.
The importance of lipoproteins for the virulence of Listeria

A study by Petit et al. (11) with a Δlgt deletion mutant of Streptococcus pneumoniae provided the first insight to the significance of prelipoprotein lipidation for virulence. It was subsequently shown that impaired lipoprotein-processing caused by deleting the lipoprotein signal peptidase gene (lsp) causes attenuation in L. monocytogenes (20) and Mycobacterium tuberculosis (25). In virulence studies with the mouse model presented here, we found similar effects using the lgt-deficient L. monocytogenes strain. Transient bacteremia, a characteristic of infection with L. monocytogenes wild-type bacteria, was almost abrogated in the case of the Δlgt mutant. A reduction in bacterial loads in spleen and liver was evident for the mutant strain throughout the infection, yet its growth kinetics in both organs was similar to the wild type. These observations reveal that lipoproteins are crucial in establishing infections in the mouse organ colonization model. We also identified an inability of the Δlgt mutant to grow in the blood of infected mice, and further studies will aim at identifying listerial lipoprotein(s) that effectively contributes to the transition of the bacterium from extracellular body fluids to cellular invasion and intracellular replication and spread.

The reduced virulence properties of the Δlgt strain were verified by performing mouse survival assays with lethal doses of bacteria. The mice infected with the deletion strain survived over a 10-day period postinfection, but all L. monocytogenes EGD-e-infected mice died within 5 days. Infection of TLR2-deficient mice results in decreased organ loads of EGD-e wild-type bacteria, supporting the observation made by Torres et al. (5) that optimal control of listerial infection requires TLR2. Bacteria lacking lipoproteins are attenuated not only in wild-type mice but also in mice deficient in TLR2 signaling. These data make it clear that the immunostimulatory properties of lipoproteins are independent of their contribution to virulence.

The in vitro experiments provide additional support for the role lipoproteins play in the virulence of L. monocytogenes. Invasion and uptake of the Δlgt strain in epithelial and macrophage cell lines, respectively, were noticeably affected. Because intracellular growth appears to be delayed rather than completely abolished, this suggests either that lipoproteins are not directly involved in this process or that the nonlipidated forms remain active. Nonetheless, several lipoproteins have been shown to take part during the early infection processes of cell entry and phagosomal escape. Among the Lgt-dependent lipoproteins characterized by Baumgärtner et al. (10), OppA and LpeA are associated with intracellular survival and bacterial entry (17), whereas the expression of three other lipoproteins is controlled by the virulence regulator PrgA (10).

To further understand the diminished virulence of the Δlgt strain, we examined bacterial resistance against cationic antimicrobial peptides and found that growth of the deletion mutant was moderately inhibited. This effect can be rationalized by either the access of these cationic peptides to the cell membrane being facilitated in the absence of lipoproteins or the anchored lipoproteins contributing to the overall charge of the bacterial surface. These data imply that lipoproteins are important for virulence by affording increased resistance against these microbial defense factors. While the exact mechanism is unknown, it is very likely that antimicrobial peptides produced by host cells during infection are factors in the reduced survival of the Δlgt strain in vivo.

Interestingly, Bubeck-Wardenburg et al. (6) presented data on an Δlgt deletion strain of S. aureus that proliferated to a higher extent compared with wild types during mouse infection. They demonstrated that S. aureus variants lacking lipoproteins are able to escape activation of an innate immune response and therefore survive better within the host. In contrast, our in vivo studies showed that the Δlgt deletion strain of L. monocytogenes is attenuated in virulence. Unlike staphylococci, Listeria organisms rely on an intracellular replication cycle to disseminate and propagate during infection, given that extracellular Listeria are cleared relatively quickly by resident macrophages and circulating leukocytes. Several factors, among them lipoproteins such as PlcB, LpeA, and PrsA are important for the invasion of nonphagocytic cells and persistence in the host (20, 26, 27). It would thus appear that the Listeria Δlgt deletion strain, which lacks lipoproteins and has a decreased ability for cell invasion, is prevented from entering the relatively secure intracytosolic environment and therefore shows reduced survival. Thus, it is clear that depending on the type of bacterium studied, the absence of TLR2-dependent recognition can have drastically different consequences for survival in the host.

In summary, this study demonstrates the requirement of bacterial lipoproteins for the early TLR2-dependent induction of the ubiquitous cellular stress signal mediator NF-κB during infection with L. monocytogenes. Our results show the importance of lipoproteins for the virulence of Listeria and for the survival of the pathogen in the animal model of infection. Further studies are now warranted to examine differences in the trajectories the immune response can take during infection in the absence of known PAMPs. The use of defined bacterial mutants that either bypass or enhance innate immune recognition by members of the TLR and Nod-like receptor families provides us with new tools to understand the contributions of the various PAMP molecules during the course of disease.

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Disclosures

The authors have no financial conflict of interest.

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