A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system

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***Listeria monocytogenes* is a human intracellular pathogen that is able to survive in the gastrointestinal environment and replicate in macrophages, thus bypassing the early innate immune defenses. Peptidoglycan (PG) is an essential component of the bacterial cell wall readily exposed to the host and, thus, an important target for the innate immune system. Characterization of the PG from *L. monocytogenes* demonstrated deacetylation of *N*-acetylglucosamine residues. We identified a PG N-deacetylase gene, *pgdA*, in *L. monocytogenes* genome sequence. Inactivation of *pgdA* revealed the key role of this PG modification in bacterial virulence because the mutant was extremely sensitive to the bacteriolytic activity of lysozyme, and growth was severely impaired after oral and i.v. inoculations. Within macrophage vacuoles, the mutant was rapidly destroyed and induced a massive IFN-β response in a TLR2 and Nod1-dependent manner. Together, these results reveal that PG N-deacetylation is a highly efficient mechanism used by *Listeria* to evade innate host defenses. The presence of deacetylase genes in other pathogenic bacteria indicates that PG N-deacetylation could be a general mechanism used by bacteria to evade the host innate immune system.**

cytokine | macrophage | pathogenesis | virulence | cell wall

The innate immune system is central for the early recognition and clearance of pathogens. Hence, a number of microbial pathogens have developed sophisticated strategies to evade or modulate the host response to their advantage. *Listeria monocytogenes* is an invasive pathogen that survives in the harsh gastrointestinal lumen and reaches the submucosa without inducing a vigorous inflammatory response. However, the mechanisms used by *L. monocytogenes* to survive during the very early steps of the infection are poorly understood. This bacterium then disseminates to various organs by spreading from cell to cell and by surviving in phagocytic cells such as macrophages. The ability of *L. monocytogenes* to internalize and escape into the cytosol requires key virulence factors such as the secreted toxin listeriolysin O (1), the surface protein ActA (2), and Internalin (3), which is anchored to its peptidoglycan (PG) (4), or InlB (5), which is attached to its lipoteichoic acids (ref. 6; for reviews see refs. 7 and 8). Hence, the cell wall of *L. monocytogenes* plays a central role in its virulence. Recently, the role of PG hydrolases in *Listeria* virulence has also been described and suggested to be important during infection (9, 10). PG is the pathogen-associated molecular pattern recognized by the innate immune system by some of the recently discovered intracellular pattern-recognition receptors such as the nucleotide-binding oligomerization domain (Nod) proteins (11). Recent work has reported a role for Nod1, Nod2 and Toll-like receptor (TLR)-2 in innate immune recognition of *Listeria* (12–15). However, little is known regarding the composition and structure of *L. monocytogenes* PG except that it is a *meso*diaminopimelic acid containing PG and that *N*-acetylglucosamine residues are partially N-deacetylated into glucosamine (16). Whether these specific features play a role in infection has never been investigated.

Here, we report that N-deacetylation is a major modification of *Listeria* PG, conferring to this human pathogen the ability to evade the innate immune system. The inactivation of the single PG N-deacetylase of *Listeria* highlights the central role of this modification in *Listeria* virulence, through survival in the gastrointestinal track, in professional phagocytes, evasion to the action of host lysozyme, and modulation of inflammatory response.

Results and Discussion

To address the role of *Listeria* PG in virulence, we first prepared highly purified PG from *L. monocytogenes* strain EGDe and analyzed its muropeptide composition by reverse-phase HPLC (Fig. 1*A*). Mass spectrometry analysis of each peak revealed that *L. monocytogenes* has several unusual modifications of its PG (Fig. 1*A*). Approximately 50% of its muropeptides featured a glucosamine residue instead of the canonical *N*-acetylglucosamine,

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Abbreviations: PEM, peritoneal macrophage; PG, peptidoglycan.

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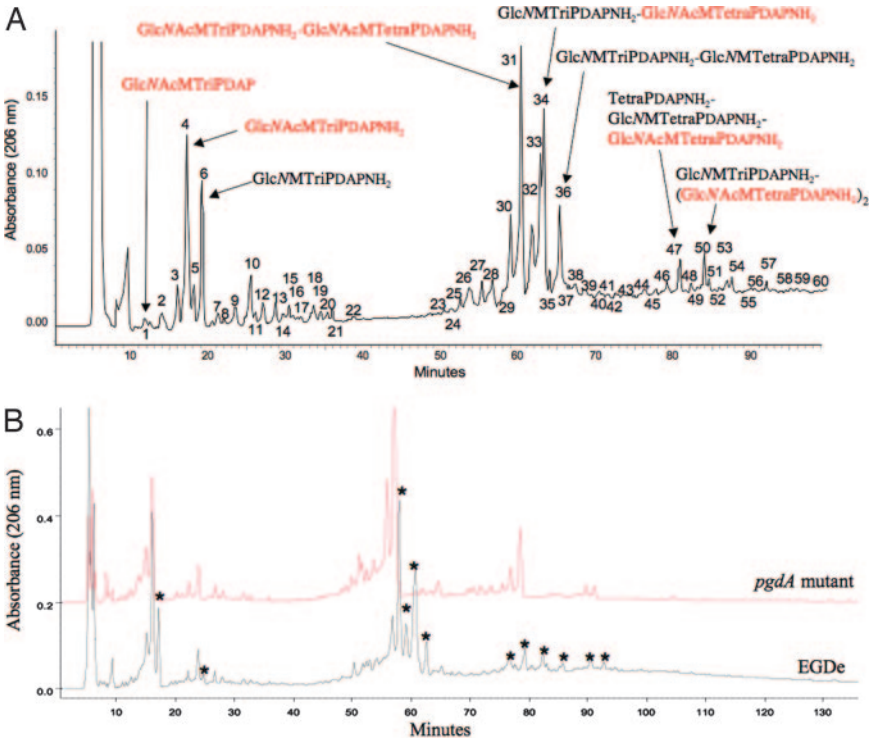

Fig. 1. Characterization of *Listeria* PG. (*A*) Each muropeptide peak highlighted by a number was purified by HPLC, desalted, and analyzed by MALDI-TOF. Structural assignment was done by muropeptide fragmentation using MALDI-postsource decay (PSD). The structure of major muropeptides is indicated by full arrows. Structures or substructures in red and black indicate fully acetylated and N-deacetylated moieties, respectively, of the different muropeptides. Peaks 1–22 correspond to monomeric muropeptides, peaks 23- 44 correspond to dimeric muropeptides, and 46 and over correspond to trimeric muropeptides. Approximately 50% of the muropeptides present a glucosamine residue instead of the canonical *N*-acetylglucosamine residue. (*B*) HPLC analysis of the muropeptide composition of *Listeria* WT EGDe strain and its *pgdA* isogenic mutant. Each muropeptide peak was purified by HPLC and analyzed by MALDI-PSD mass spectrometry. Muropeptide peaks indicated with an asterisk correspond to N-deacetylated muropeptides. N-deacetylated muropeptides characteristic of the parental strain EGDe were completely absent from the elution pattern of the *pgdA* mutant. Glc*N*Ac, *N*-acetylglucosamine; Glc*N*, glucosamine; M, *N*-acetylmuramic acid, TriPDAP, L-alanyl-γ-D-glutamyl-*meso*diaminopimelic acid; TriPDAPNH$_2$, L-alanyl-γ-D-glutamyl-amidated *meso*diaminopimelic acid; TetraPDAP, L-alanyl-γ-D-glutamyl-*meso*diaminopimelyl-D-alanine; TetraPDAPNH$_2$, L-alanyl-γ-D-glutamyl-amidated *meso*diaminopimelyl-D-alanine.

definitively establishing that *Listeria* partially N-deacetylates its PG. Interestingly, PG N-deacetylation has been described as conferring resistance to host lysozyme (17), a major innate defense mechanism against bacterial infections.

Analysis of the *L. monocytogenes* genome allowed identification of a gene encoding a putative PG deacetylase (*lmo0415*), the only putative PG N-deacetylase that could be identified. A deletion mutant of *lmo0415* was generated. Analysis of its PG confirmed that *lmo0415*, renamed *pgdA*, encodes the unique PG N-deacetylase of *L. monocytogenes* because all peaks corresponding to N-deacetylated muropeptides disappeared from the HPLC pattern (Fig. 1*B*). As expected, the *pgdA* mutant showed sensitivity to lysozyme, resulting in a five-log decrease in viability compared with the parental strain EGDe in the presence of lysozyme (Fig. 2). Interestingly, the sensitivity of the *pgdA* mutant was specific for lysozyme, because the human serum amidase, another PG hydrolase produced by the host, had no bacteriolytic effect on the *pgdA* mutant (Fig. 2*A*). Also, the mutant was perfectly capable of growing in BHI broth and serum, indicating that *pgdA* inactivation had no effect on its growth [Fig. 2*A* and supporting information (SI) Fig. 6] or morphology (SI Fig. 7). Sensitivity to lysozyme was observed only in stationary phase and resulted in cell rounding (SI Fig. 7).

As shown above, the *pgdA* mutant is extremely sensitive to host lysozyme. Because lysozyme is particularly abundant in macro-

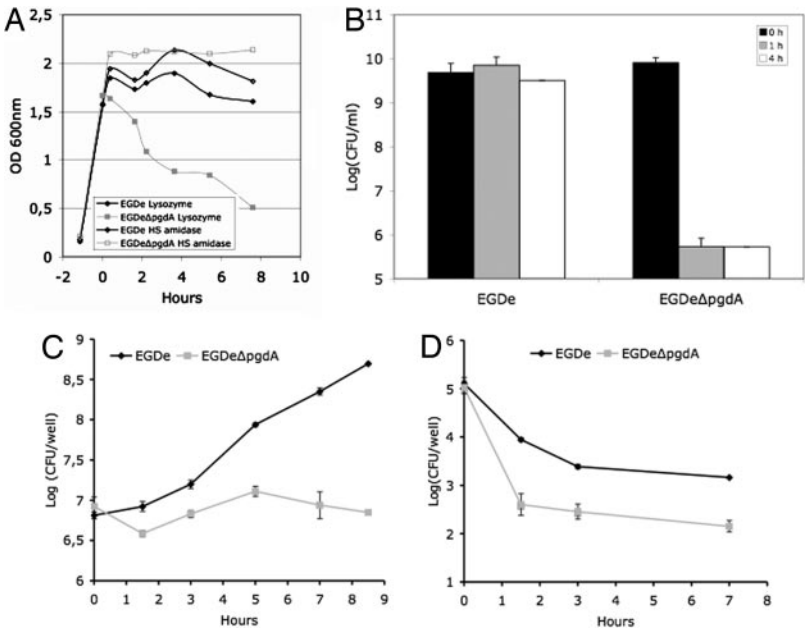

Fig. 2. Effect of lysozyme on growth and impaired survival in macrophages of the *pgdA* mutant. Strain EGDe and its isogenic *pgdA* mutant were grown in BHI media and incubated with lysozyme (10 μg/ml) or the human serum amidase (1 μg/ml). (*A*) The *pgdA* mutant was selectively sensitive to the action of lysozyme upon entry into stationary phase. (*B*) Decrease of the optical density of the *pgdA* mutant correlated with a loss of viability, whereas the parental strain was insensitive to lysozyme. Lysozyme induced cell rounding of the *pgdA* mutant (see SI Fig. 7). (*C* and *D*) RAW264.7 macrophages (*C*) and PEM (*D*) were infected with WT EGDe and its *pgdA* mutant. Sensitivity of the *pgdA* mutant to lysozyme correlated with its impaired survival in macrophages.

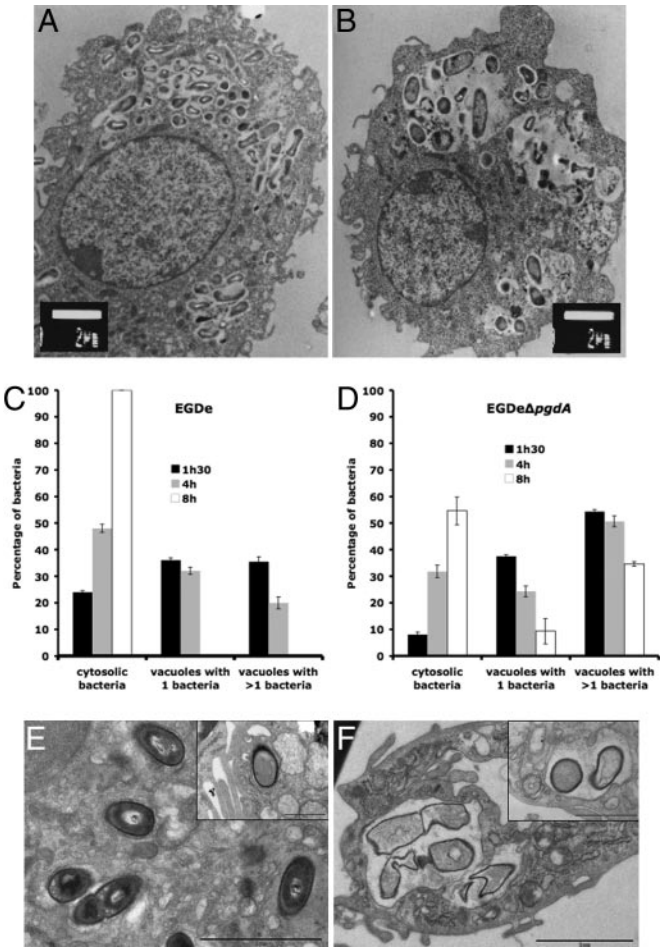

Fig. 3. Impaired survival of the *pgdA* mutant in macrophages. (*A–D*) RAW264.7 cells after 8 h of infection with the parental strain EGDe (*A*) and the *pgdA* mutant (*B*). Impaired survival was correlated with delay in escape of the *pgdA* mutant (*C*) from phagosomes compared with the WT strain EGDe (*D*). (*E* and *F*) PEM after 7 h of infection with the parental strain EGDe (*E*) and the *pgdA* mutant (*F*). [Scale bars: 2 μm and 1 μm (*Insets*).] Impaired survival correlated with delay in escape from phagosomes and bacterial lysis of the *pgdA* mutant compared with the WT strain EGDe.

phages, to evaluate the possible impact of N-deacetylation on infection, we compared the ability of the *pgdA* mutant and that of strain EGDe to infect and survive in macrophages (Fig. 2 *C* and *D*). The *pgdA* mutant was severely impaired in its ability to survive and multiply in RAW264.7 macrophages (Fig. 2*C*), in peptone-elicited peritoneal macrophages (PEM) from C57/BL6J mice (Fig. 2*D*) and in bone marrow-derived macrophages from C57/BL6J mice (data not shown). For example, after 8 h of infection of RAW264.7 macrophages, the difference in viable bacteria between the *pgdA* mutant and strain EGDe was 1.5 log. Next, infected macrophages were observed by EM. Fig. 3 *A* and *B* illustrates EM images of macrophages infected with strain EGDe and the *pgdA* mutant, respectively, after 8 h of infection (see also Fig. 3 *E* and *F*). Whereas strain EGDe was present exclusively in the macrophage cytosol, the *pgdA* mutant accumulated in vacuoles, preventing its cytosolic multiplication. EM observations of infected macrophages at various time points after infection showed that the *pgdA* mutant was mainly detected in vacuoles compared with the parental strain EGDe (Fig. 3 *C* and *D*), suggesting an early intracellular killing. Strikingly, the *pgdA* mutant was found frequently in large vacuoles with multiple bacteria or bacterial debris (Fig. 3 *A*, *B*, *E*, and *F*). We also infected nonphagocytic Caco 2 epithelial cells with strain EGDe and its *pgdA* mutant. The *pgdA* mutant was not impaired in its ability to be internalized (data not shown), indicating that the *pgdA* mutant was not generally affected in bacterial stability. Moreover, the *pgdA* mutant produced normal amounts in listeriolysin O, showing that impaired escape from phagosomes in macrophages is not due to a defect of listeriolysin O production (data not shown).

Virulence and dissemination of *Listeria* in its host strongly relies on survival in macrophages, as illustrated by the very strong attenuation of the listeriolysin O mutant (1). We thus performed *in vivo* challenge of BALB/c and C57/BL6J mice by the i.v. route with strain EGDe and the *pgdA* mutant. In both mouse backgrounds, the *pgdA* mutant was severely attenuated, with LD_{50}s of 1.7×10^6 and 1.1×10^8 bacteria in C57/BL6J and BALB/c mice, respectively, compared with 2.3×10^4 and 1.7×10^4 bacteria, respectively, for the parental strain EGDe. Accordingly, bacterial counts for the *pgdA* mutant were lower than the WT in both liver and spleen (Fig. 4 *A* and *B*). In addition, the *pgdA* mutant, although sensitive to lysozyme *in vitro*, was as resistant as the WT strain to the action of serum in the presence or absence of complement (SI Fig. 6). Hence, the impaired virulence of the *pgdA* mutant is not related to an impaired survival in the blood stream.

The oral route is the natural route of *Listeria* infection. We thus also orally infected human E-cadherin (hEcad) transgenic mice, which are permissive to *Listeria* oral infection (18), and monitored bacterial counts in different organs at 3, 24, 48, and 72 h after infection (Fig. 4 *C–G*). The *pgdA* mutant was strongly impaired in surviving in the intestinal lumen, and, 48 h after infection, no bacteria could be detected in this compartment (Fig. 4*C*). Furthermore, bacterial counts of the *pgdA* mutant were compared with the WT. Those were lower in the intestine and in the mesenteric lymph nodes (Fig. 4 *D* and *E*, respectively) and, as observed after IV inoculation, in the liver and the spleen (Fig. 4 *F* and *G*, respectively). Hence, the *pgdA* mutant is attenuated in virulence at both very early stages of the infection and at later stages after dissemination from the intestine to the liver and the spleen. These results reveal that the *pgdA* mutant is highly sensitive to both the very first host innate immune responses and at later stages of the infection. This finding is in agreement with the fact that Paneth cells in the small intestine abundantly produce lysozyme and other antimicrobial agents.

We then investigated the impact that lack of PG N-deacetylation might have on the inflammatory response. Because the Nod proteins detect PG and PG fragments such as muropeptides (19, 20), we first analyzed the ability of highly purified PG from *L. monocytogenes* EGDe to activate the Nod pathways by measuring NF-κB activation with the classical luciferase reporter assay in HEK293T cells (21). The native PG of *L. monocytogenes* activated NF-κB poorly in both Nod1- and Nod2-dependent manners compared with *Escherichia coli* PG (SI Fig. 8) as reported (22). However, a complete predigestion with a muramidase increased induction of NF-κB in a Nod2-dependent (20-fold) and Nod1-dependent (6-fold) manner (SI Fig. 8*A*). The fully acetylated native PG from the *pgdA* mutant was better detected by the Nods (SI Fig. 8*B*), compared with the native PG of parental strain EGDe. Taken together, our results suggested that *L. monocytogenes* contains in its native PG Nod agonists but that these are not readily available to the host. Hence, our hypothesis that *Listeria* also N-deacetylates its PG as a strategy to avoid generation and presentation of cell-wall components to the pattern-recognition receptors such as the Nods or TLR2. As a proof of principle, we were able to show that an *in vitro* fully N-deacetylated PG from *Helicobacter pylori* completely lost its ability to be sensed by both Nod1 and Nod2 (SI Fig. 8*C*) compared with native *H. pylori* PG.

The cytokine response of macrophages to the *pgdA* mutant was then compared with WT. Despite the reduced number of viable mutant bacteria (see Fig. 3 *A* and *B*), the *pgdA* mutant induced a strikingly more vigorous cytokine response compared with WT (for example, higher amounts of IL-6 and IFN-β) than strain EGDe in RAW264.7 macrophages (Fig. 5

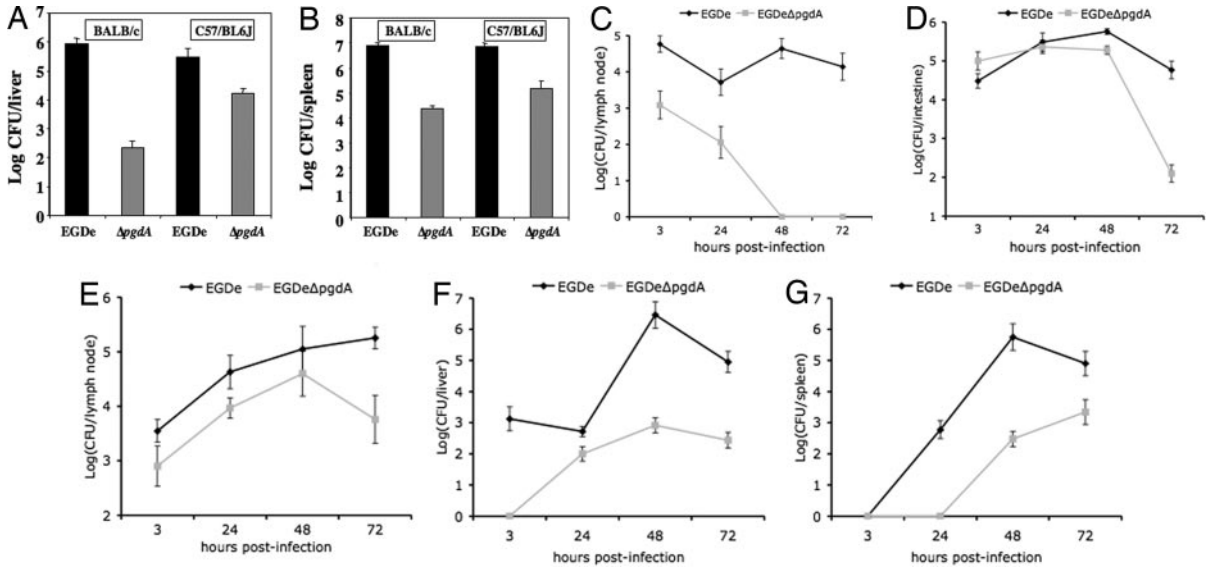

Fig. 4. Impaired virulence of the *pgdA* mutant *in vivo*. (*A* and *B*) BALB/c and C57/BL6J mice were challenged by i.v. injection with the parental strain EGDe and its *pgdA* mutant with a sublethal dose (5×10^3 CFU per mouse). After 72 h, mice were killed, and bacterial counts in the liver (*A*) and the spleen (*B*) were determined. (*C–G*) Transgenic human E-cadherin mice were used as model for the oral route of infection, and colonization of several organs was followed after 3, 24, 48, and 72 h after challenge. Interestingly, the *pgdA* mutant was particularly vulnerable to persistence in the intestinal lumen as assayed by bacterial counts per grams of feces (*C*). Survival was more robust in the intestine (*D*) and the mesenteric lymph nodes (*E*) where colonization was particularly impaired after 72 h. The mutant was impaired in the survival of the liver (*F*) and spleen (*G*) at all time points as in the IV model shown in *A* and *B*.

A and *B* and SI Fig. 9). This enhanced cytokine response of the *pgdA* mutant compared with WT EGDe was also observed in PEM (Fig. 5 *C* and *D*, respectively). To our knowledge, the stimulation of IFN-β production by PEM at such a high level by the *pgdA* mutant is previously undescribed in *Listeria*.

The role of different pattern-recognition receptors in the enhanced cytokine response to *pgdA* mutant infection was then addressed by using macrophages from Nod1$^{-/-}$, Nod2$^{-/-}$, MyD88$^{-/-}$, and TLR2$^{-/-}$ mice. IL-6 and IFN-β production depended strongly on TLR2. The TLR2 response depended partially on MyD88, the master adaptor molecule myeloid differentiation factor 88. IL-6 production also partially depended on Nod1, which, surprisingly, negatively regulated IFN-β production by PEM (Fig. 5*D*). This negative regulatory impact on TLR2-mediated IFN-β production of the Nod1-pathway is reminiscent of the role of Nod2 as negative regulator of TLR2-mediated T helper type 1 responses (23). Together, our results suggested that the inflammatory response induced by the *pgdA* mutant is due to an enhanced accessibility of cell wall components to TLR2 and Nod1. These results also suggested, as shown in PEM, that WT *Listeria* exploit PG N-deacetylation to evade Nod1 sensing.

In conclusion, the *pgdA* mutant is a highly attenuated mutant of *Listeria*. Attenuation is in the same order of magnitude as that of listeriolysin O and ActA mutants, the most attenuated *Listeria* mutants (7, 8). Thus, N-deacetylation of *Listeria*'s PG is a major virulence determinant. *Listeria* escapes recognition and killing by

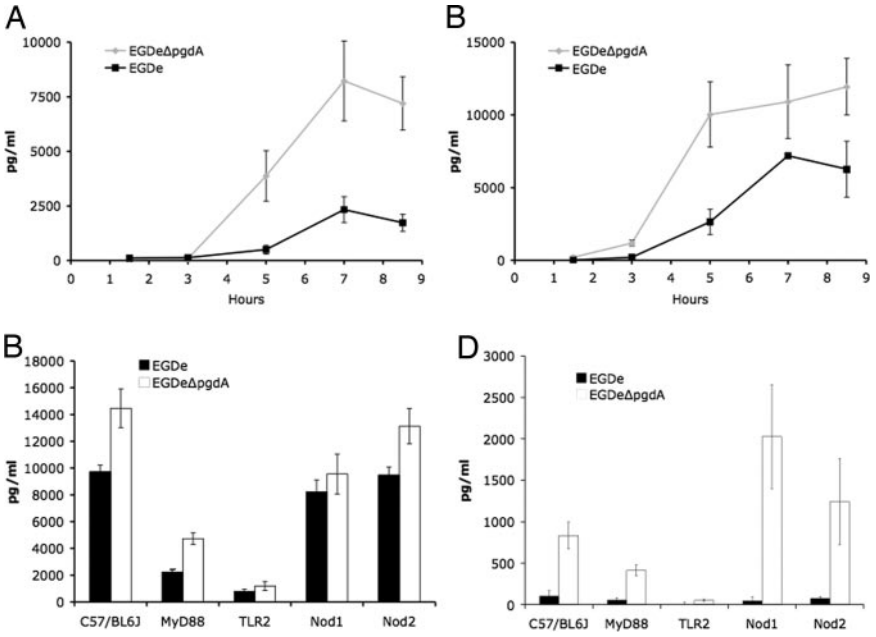

Fig. 5. Enhanced inflammatory response of the *pgdA* mutant. Cytokine production such as IL-6 (*A*) and IFN-β (*B*) by RAW264.7 macrophages was enhanced by the *pgdA* mutant compared with the parental strain EGDe (see also SI Fig. 9). PEM of WT C57/BL6J, Nod1$^{-/-}$, Nod2$^{-/-}$ TLR2$^{-/-}$ and MyD88$^{-/-}$ mice were infected with strains EGDe and its isogenic *pgdA* mutant. After 7 h of infection, the inflammatory response was enhanced as measured by the amount of IL-6 (*C*) and, particularly, IFN-β (*D*) production. The cytokine response depended mainly on TLR2 and Myd88, although their contribution varied according to the cytokine measured. IFN-β production depended almost entirely on TLR2 in a MyD88-dependent and -independent manner (*D*). IL-6 production depended only partially on TLR2 and Myd88 (*C*) and also required Nod1. Surprisingly, Nod1 seemed to function as an inhibitor of IFN-β production, because the *pgdA* mutant induced 3-fold more IFN-β in Nod1$^{-/-}$ compared with C57/BL6J peritoneal macrophages (*D*). Note that the role of Nod1 is entirely restricted to the response to the *pgdA* mutant and does not participate in the response to the WT EGDe strain.

the host by preventing processing and optimal sensing of its PG by the host. Our observations *in vitro* and *in vivo* indicate that a major consequence of PG N-deacetylation is an increased survival at early and also later stages of the infectious process. We propose the following model (SI Fig. 10). *Listeria* PG N-deacetylation enhances the pathogen's ability to survive to first defenses of the host in the intestinal lumen by avoiding the bacteriolytic activity of lysozyme that is massively produced by Paneth cells in the intestine (24). *Listeria* PG N-deacetylation might also prevent induction of enhanced production of antimicrobial peptides synthesis by escaping Nod2 detection in the intestine as previously proposed, thus potentially coupling resistance to lysozyme to the Nod2 evasion (12). From the primary infection site, *Listeria* disseminates to target organs, owing to escape from lysozyme, survival in phagocytic cells, and down-regulation of the inflammatory response. The *pgdA* mutant sensitivity to lysozyme results in its enhanced destruction by phagocytes and release of its cell-wall components such as muropeptides and lipoteichoic acid (LTA). The *pgdA* mutant's LTA is readily available to membrane-bound TLR2, whereas muropeptides can be delivered to the cytosol by endogenous transporters such as hPepT1 (25) or after pore formation by listeriolysin O. Cytokine production would then ensue. PG N-deacetylation would thus function as a double-protection mechanism for *Listeria* against the innate immune defenses by escaping the action of lysozyme and providing a mechanism to evade TRL2 and the Nod proteins. The predominant TLR2-dependent phenotype is most probably due to the extreme sensitivity of the *pgdA* mutant to the phagosome environment before any major contribution of the cytosolic sensing mechanism by the Nods can occur. In perfect agreement with this report, N-deacetylation of pneumococcal PG had been associated with pneumococcal virulence, but the underlying mechanisms had not been studied in detail (26). PG N-deacetylation also occurs in other major Gram-positive pathogens such as *Bacillus anthracis*. Genome analysis of *B. anthracis* indicates the presence of 10 ORFs of putative deacetylases (27), suggesting that these might contribute to extreme virulence of this bioterrorism agent. Enterobacteria such as *E. coli*, *Shigella flexneri*, and *Yersinia pestis* have an unique lysozyme inhibitor, the small periplasmic polypeptide Ivy (28). Alternatively, *Staphylococcus aureus*, *Neisseria meningitidis*, and *Mycobacterium tuberculosis* have developed mechanisms such as PG O-acetylation and PG N-glycolylation of their muramic acid residues to counteract the activity of host lysozyme (29–31), suggesting that these major pathogens might also modulate the host innate immune response by modifications of their PGs. Our study reveals a mechanism by which pathogens modify their PG to escape from host defenses.

Materials and Methods

Bacterial and Cell Growth Conditions. *E. coli* DH5α was used as host for the construction and preparation of plasmids. *E. coli* was cultivated in Luria Bertani solid or liquid media supplemented as appropriate with ampicillin (100 $\mu g \cdot ml^{-1}$). *E. coli* MC1061 (53338; American Type Culture Collection, Manassas, VA) (32) and *H. pylori* strain 26695 (700392; American Type Culture Collection) (33) were used to extracted PG. *H. pylori* was grown microaerobically at 37°C on blood agar plates or in liquid medium consisting of brain-heart infusion (BHI; Oxoid, Basingstock, Hampshire, U.K.) with 0.2% β-cyclodextrin (Sigma) supplemented with antibiotic–antifungal mix (34). *L. monocytogenes* EGDe (BUG1600; BAA-679; American Type Culture Collection) and its *pgdA* mutant (BUG 2288) were grown in BHI solid or liquid media. HEK293T (CRL-1573; American Type Culture Collection) cells and RAW264.7 (TIB-71; American Type Culture Collection) macrophages were cultured in DMEM (GIBCO, Paisley, Scotland, U.K.) containing 10% FCS (GIBCO). Before transfection, HEK293T cells were seeded into 24-well plates at a density of 10^5 cells per ml as described (35). RAW264.7 macrophages were seeded into either six-well plates or into 25-mm Petri dishes at 10^5 cells per ml and 10^6 cells per ml, respectively.

Construction of *Listeria pgdA* Mutant. A DNA fragment containing 663 bp of the sequence upstream of *lmo0415* was generated by PCR using oligonucleotides *lmo0415–1* (5′-AACAGGATCCATAACTGGAGACACGGAGAC-3′) and *lmo0415–2* (5′-AACAGAATTCATTATGCACCTCACCTCAG-3′). The fragment was cloned into BamHI-EcoRI digested *pMAD* (36). A DNA fragment containing 702 bp of the sequence downstream of *lmo0415* was generated by PCR using oligonucleotides *lmo0415–3* (5′-AACAGAATTCAAATCAGTAGCTAAGATGAGTTAA-3′) and *lmo0415–4* (5′-AACAAGATCTGATTGTCAAACTTGAAATGG-3′). The fragment was cloned into EcoRI-BglII-digested *pMAD* containing the *lmo0415* upstream fragment, constructing the pDC27. The construct was verified by sequencing. To achieve allelic exchange, pDC27 was electroporated into *L. monocytogenes* EGDe at 2,500 V, 200 Ω ,and 25 μF. Transformants were selected at 30°C on BHI agar medium containing erythromycin (5 μg/ml) and X-Gal (50 μg/ml). One blue colony was grown in BHI-Ery broth at 43.5°C for 48 h, and the culture was plated onto BHI-Ery agar containing X-Gal at 43.5°C. One blue colony was selected and grown in BHI broth at 30°C. BHI broth was inoculated with 1 μl of a 1:10 dilution of the previous culture and incubated at 43.5°C. Tenfold serial dilutions of this culture were plated onto BHI-X-Gal agar and incubated at 43.5°C. White colonies were analyzed for erythromycin sensitivity, and PCR amplifications with oligonucleotides *lmo0415–1* and *lmo0415–4* and oligonucleotides *lmo0415–5* (5′-GATGGACAGACTAATGAAAGACC-3′) and *lmo0415–6* (5′-AAAGCACCTGTTTCTGCGTC-3′) were performed to confirm the gene deletion.

Lysozyme, PGRP-L, Serum Experiments, and Hemolytic Activity. *Listeria* strains were grown in BHI media to which was added either hen egg lysozyme (10 μg/ml final concentration; Sigma-Aldrich), human serum amidase (or PGRP-L, kindly provided by Waldemar Vollmer, University of Tübigen, Tübigen, Germany; 1 μg/ml final concentration) or FCS (final concentration at 25%; GIBCO). When needed, decomplementation was done by heating the FCS at 65°C for 30 min. Growth was monitored by following the optical density at 600 nm and by determining the number of viable bacteria per milliliter. *L. monocytogenes* strains were grown on 5% horse blood agar plates. Plates were incubated at 37°C for 48 h and at 4°C for 24 h. The size of the clear zone around the colonies was measured, which indicated β-hemolytic activity.

PG Purification, HPLC Analysis, *in Vitro N*-Deacetylation and MALDI-postsource decay (PSD) Analysis. *E. coli*, *L. monocytogenes*, and *H. pylori* PGs were prepared from exponentially growing bacteria and purified as described (22). From native PG, muropeptides were generated by using the muramidase mutanolysin M1 (Sigma-Aldrich), separated by HPLC, purified, and analyzed by MALDI-PSD as described (31). N-deacetylation of *H. pylori* PG was done as described by using the recombinant BC1960 deacetylase from *Bacillus cereus* (27).

Expression Plasmids, Transient Transfections, and NF-κB Activation Assays. HEK293T cells were used for transfections as described (35). Synergistic activation of NF-κB by PGs, muramyl peptides, and related compounds in cells overexpressing Nod1 or Nod2 was studied as described by Inohara *et al.* (21). Data were standardized with positive controls: *N*-acetylmuramic acid-dipeptide for hNod2 and *N*-acetylmuramic acid-tripeptide for hNod1. hNod1 and hNod2 were activated with PG (0.3 μg/ml) as described (38). More detailed information is available in *SI Text*.

Infection of Murine Macrophages. PEM and bone marrow-derived macrophages (BMDM) were isolated from BALB/c, C57/BL6J

(Charles River Laboratories, L'Arbresle, France), TLR2$^{-/-}$, MyD88$^{-/-}$, Nod1$^{-/-}$, Nod2$^{-/-}$ mice. BMDM preparation is detailed in *SI Text*. PEM were established as described (39). PEM or BMDM (10^6 cells per well) were infected with *L. monocytogenes* strains (MOI, 10), grown to an OD_{600} of 0.8, and incubated at 37°C for 1 h to allow bacterial phagocytosis. Extracellular bacteria were eliminated by three washes in RPMI medium 1640 supplemented with 10% FCS, and 10 μg/ml gentamicin was added for the time of infection. Macrophages were lysed with 0.2% Triton X-100 at various time points (1.5, 3, and 7 h) for 10 min, and the number of CFU was assessed by plating serial dilutions on BHI agar plates that were incubated at 37°C.

Epithelial Caco 2 Cells and RAW264.7 Macrophage Infections. The *Listeria* strains were grown to an OD_{600} of 0.6–0.8, washed three times, and diluted in DMEM such that the MOI was ≈100 and 25 bacteria per Caco-2 cell and RAW264.7 macrophage, respectively (OD_{600} of 1 corresponds to $\approx 10^9$ bacteria). Bacterial suspensions were added to mammalian cells for 1 h, and the cells were then washed and noninvasive bacteria were killed by adding 20 μg/ml gentamicin for 1.5 h. After washing three times, cells were lysed in 0.2% Triton X-100 at various desired time points (0, 1.5, 3, 5, 7, and 8.5 h), and the number of viable bacteria released from the cells was assessed after serial dilutions of the lysates on BHI agar plates. Supernatants were collected at the various time points to determine cytokine production. For EM observations, infected cells were recovered and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C.

EM. Fixed samples were rinsed three times with 0.1 M cacodylate buffer, postfixed with 2% osmium tetraoxide, 1% potassium ferricyanide in 0.1 M cacodylate buffer for 1 h at room temperature, and washed again with 0.1 M cacodylate buffer and water. The samples were dehydrated through a graded series of ethanol baths, one time with propylene oxide, and overnight in a mixture of Epon 812/propylene oxide at room temperature. After being embedded in Epon 812, samples were polymerized for 48 h at 60°C. Utrathin sections (70–80 nm) were cut with a diamond knife, stained with uranyl acetate and Reynolds lead citrate, and viewed in a JEM 1010 transmission electron microscope (Jeol, Tokyo, Japan) at 80 kV by using an Eloïse Mega View III camera and AnalySIS Pro Software version 3.1 (comEloïse SARL, Roissy, France).

Mouse Experiments. Male mice 6 to 10 weeks old were used for this study. Detailed information on mice backgrounds is available in *SI Text*. LD50 were performed after i.v. challenge of mice with increasing doses of WT EGDe and its *pgdA* mutant. Survival was monitored during 3 weeks, and LD_{50} were calculated as described (40). Kinetics of infection was monitored i.v. in BALB/C or C57/BL6J mice at 72 h after challenge with a sublethal dose of strain EGDe and its *pgdA* mutant (5×10^3 bacteria per mouse). Oral infections were performed as described (18). Female *iFABP-hEcad* transgenic mice (6 to 8 weeks old) starved for 24 h were injected intragastrically with 5×10^9 bacteria mixed with PBS 150 mg/ml CaCO3. At 3, 24, 48, or 72 h after infection, the organs were dissected. The small intestine was rinsed and incubated for 2 h in 100 mg/liter gentamicin to kill extracellular bacteria from the intestinal lumen. Monitoring of *Listeria* in the intestinal lumen was done by determining the bacterial load of *Listeria* per gram of feces by using a *Listeria*-selective media (Oxford media; Oxoid).

Cytokine Production. Supernatants of macrophages (RAW264.7 and PEM) infected by strain EGDe and isogenic *pgdA* mutant were used to determine the production of IL-6, IL-1β, TNF-α, and IFN-β using the Quantikine mouse IL-6, IL-1β, and TNF-α (R & D Systems, Minneapolis, MN) and mouse IFN β (PBL Biochemical Laboratories, Piscataway, NJ) ELISA kits, respectively, as recommended by the manufacturers.

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1. Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Berche P (1989) *Infect Immun* 57:3629–3636.
2. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P (1992) *Cell* 68:521–531.
3. Gaillard JL, Berche P, Frehel C, Gouin E, Cossart P (1991) *Cell* 65:1127–1141.
4. Lebrun M, Mengaud J, Ohayon H, Nato F, Cossart P (1996) *Mol Microbiol* 21:579–592.
5. Dramsi S, Biswas I, Maguin E, Braun L, Mastroeni P, Cossart P (1995) *Mol Microbiol* 16:251–261.
6. Jonquieres R, Bierne H, Fiedler F, Gounon P, Cossart P (1999) *Mol Microbiol* 34:902–914.
7. Dussurget O, Pizarro-Cerda J, Cossart P (2004) *Annu Rev Microbiol* 58:587–610.
8. Hamon M, Bierne H, Cossart P (2006) *Nat Rev Microbiol* 4:423–434.
9. Cabanes D, Dussurget O, Dehoux P, Cossart P (2004) *Mol Microbiol* 51:1601–1614.
10. Lenz LL, Mohammadi S, Geissler A, Portnoy DA (2003) *Proc Natl Acad Sci USA* 100:12432–12437.
11. Meylan E, Tschopp J, Karin M (2006) *Nature* 442:39–44.
12. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA (2005) *Science* 307:731–734.
13. Opitz B, Puschel A, Beermann W, Hocke AC, Forster S, Schmeck B, van Laak V, Chakraborty T, Suttorp N, Hippenstiel S (2006) *J Immunol* 176:484–490.
14. Torres D, Barrier M, Bihl F, Quesniaux VJ, Maillet I, Akira S, Ryffel B, Erard F (2004) *Infect Immun* 72:2131–2139.
15. Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA (2002) *Nature* 416:194–199.
16. Kamisango K, Saiki I, Tanio Y, Okumura H, Araki Y, Sekikawa I, Azuma I, Yamamura Y (1982) *J Biochem (Tokyo)* 92:23–33.
17. Amano K, Hayashi H, Araki Y, Ito E (1977) *Eur J Biochem* 76:299–307.
18. Lecuit M, Vandormael-Pournin S, Lefort J, Huerre M, Gounon P, Dupuy C, Babinet C, Cossart P (2001) *Science* 292:1722–1725.
19. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D (2003) *J Biol Chem* 278:41702–41708.
20. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, *et al.* (2003) *J Biol Chem* 278:5509–5512.
21. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G (2001) *J Biol Chem* 276:2551–2554.
22. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, Boneca IG (2004) *EMBO Rep* 5:1000–1006.
23. Watanabe T, Kitani A, Murray PJ, Strober W (2004) *Nat Immunol* 5:800–808.
24. Dommett R, Zilbauer M, George JT, Bajaj-Elliott M (2005) *Mol Immunol* 42:903–912.
25. Vavricka SR, Musch MW, Chang JE, Nakagawa Y, Phanvijhitsiri K, Waypa TS, Merlin D, Schneewind O, Chang EB (2004) *Gastroenterology* 127:1401–1409.
26. Vollmer W, Tomasz A (2002) *Infect Immun* 70:7176–7178.
27. Psylinakis E, Boneca IG, Mavromatis K, Deli A, Hayhurst E, Foster SJ, Varum KM, Bouriotis V (2005) *J Biol Chem* 280:30856–30863.
28. Monchois V, Abergel C, Sturgis J, Jeudy S, Claverie JM (2001) *J Biol Chem* 276:18437–18441.
29. Bera A, Herbert S, Jakob A, Vollmer W, Gotz F (2005) *Mol Microbiol* 55:778–787.
30. Raymond JB, Mahapatra S, Crick DC, Pavelka MS, Jr (2005) *J Biol Chem* 280:326–333.
31. Antignac A, Rousselle JC, Namane A, Labigne A, Taha MK, Boneca IG (2003) *J Biol Chem* 278:31521–31528.
32. Casadaban MJ, Cohen SN (1980) *J Mol Biol* 138:179–207.
33. Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, *et al.* (1997) *Nature* 388:539–547.
34. Bury-Mone S, Thiberge JM, Contreras M, Maitournam A, Labigne A, De Reuse H (2004) *Mol Microbiol* 53:623–638.
35. Girardin SE, Tournebize R, Mavris M, Page AL, Li X, Stark GR, Bertin J, DiStefano PS, Yaniv M, Sansonetti PJ, Philpott DJ (2001) *EMBO Rep* 2:736–742.
36. Arnaud M, Chastanet A, Debarbouille M (2004) *Appl Environ Microbiol* 70:6887–6891.
37. Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, Carrio R, Merino J, Liu D, Ni J, Nunez G (1999) *J Biol Chem* 274:14560–14567.
38. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, Tedin K, Taha MK, Labigne A, Zahringer U, *et al.* (2003) *Science* 300:1584–1587.
39. Alford CE, King TE, Jr, Campbell PA (1991) *J Exp Med* 174:459–466.
40. Reed LJ, Muench H (1938) *Am J Hyg* 27:493–497.