OatA, a Peptidoglycan O-Acetyltransferase Involved in *Listeria monocytogenes* Immune Escape, Is Critical for Virulence

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Microbial pathogens have evolved mechanisms to overcome immune responses and successfully infect their host. Here, we studied how *Listeria monocytogenes* evades immune detection by peptidoglycan (PGN) modification. By analyzing *L. monocytogenes* muropeptides, we detected *O*-acetylated muramic acid residues. We identified an *O*-acetyltransferase gene, *oatA*, in the *L. monocytogenes* genome sequence. Comparison of PGN from parental and isogenic *oatA* mutant strains showed that the *O*-acetyltransferase OatA O-acetylates *Listeria* PGN. We also found that PGN *O*-acetylation confers resistance to different types of antimicrobial compounds targeting bacterial cell wall such as lysozyme, β-lactam antibiotics, and bacteriocins and that *O*-acetylation is required for *Listeria* growth in macrophages. Moreover, *oatA* mutant virulence is drastically affected in mice following intravenous or oral inoculation. In addition, the *oatA* mutant induced early secretion of proinflammatory cytokines and chemokines in vivo. These results suggest an important role for OatA in limiting innate immune responses and promoting bacterial survival in the infected host.

Listeria monocytogenes is a Gram-positive bacterium widespread in the environment and the etiologic agent of listeriosis, a life-threatening food-borne disease. Clinical manifestations of the disease range from gastroenteritis to septicemia, central nervous system infections, abortions, and perinatal infection [1]. After ingestion of contaminated food, *L. monocytogenes* can survive in the intestine, cross the intestinal barrier, disseminate to the liver and spleen, and ultimately spread to the central nervous system or fetoplacental unit. Invasive listeriosis relies on the *Listeria* capacity to enter and replicate in phagocytes and nonphagocytic cells such as epithelial cells, endothelial cells, and hepatocytes [2]. After internalization in a vacuole, *Listeria* secretes listeriolysin O (LLO), a pore-forming toxin essential for virulence that promotes bacterial escape from the vacuole [3]. In the cytosol, *Listeria* replicates and exploits the actin polymerization machinery to propel itself, escape control by autophagy, and spread from cell to cell, protected from extracellular immune effectors such as complement and antibodies.

Innate immune response is critical to controlling *Listeria* infection [4, 5]. Neutrophils are major effectors of the innate response, attracted to the liver to eliminate hepatocytes infected by *Listeria* [6, 7]. Activation of neutrophils relies on cytokines, notably interleukin 6 (IL-6) [8]. IL-6–deficient mice have a defective neutrophil response and are highly susceptible to listeriosis [9, 10]. Macrophages and natural killer (NK) cells are also essential for *Listeria* clearance, and cytokines are central to their activation. Secretion of tumor

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necrosis factor–α (TNF-α) and interleukin 12 (IL-12) by macrophages induces interferon-γ (IFN-γ) release by NK cells [11], which activate macrophage bactericidal activity in mice infected by *Listeria* [12]. TNF-α and IFN-γ are important for resistance to listeriosis, as mice that lack TNF-α receptor or IFN-γ fail to eradicate *Listeria* and rapidly die [13–18].

Pathogenic bacteria have a variety of mechanisms to subvert innate immunity [19]. We previously showed that *Listeria* evades innate defenses by peptidoglycan (PGN) *N*-deacetylation [20]. PGN is an essential component of bacterial cell wall and a pathogen-associated molecular pattern that activates pattern-recognition receptors (PRRs; eg, nucleotide-binding oligomerization domain [NOD] proteins), which trigger antimicrobial signaling cascades and pathogen clearance [21]. Modification of PGN *N*-acetylglucosamine residues by *Listeria* deacetylase PgdA confers resistance to lysozyme, a major antibacterial of the innate immune system [20]. Indeed lysozyme, by hydrolyzing PGN β-(1,4)-glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid residues, has a bactericidal activity and enhances presentation of pathogen-associated molecular patterns (PAMPs) to PRRs [22]. Accordingly, *Listeria* PGN deacetylation suppresses NOD1-dependent and toll-like receptor 2 (TLR2)–dependent IL-6 and interferon-β secretion, possibly by decreasing accessibility of cell wall components to PRRs [20]. *Listeria* PGN has not been fully characterized yet. Thus, it is currently unknown whether deacetylation of *N*-acetylglucosamine residues is the only PGN modification evolved by *Listeria* to evade innate immunity.

Here, we report that *Listeria* evades innate defenses by PGN *O*-acetylation. We show that *L. monocytogenes* PGN *O*-acetyltransferase OatA confers resistance to lysozyme and other antibacterials. Furthermore, we demonstrate that OatA contributes not only to intracellular survival in macrophages in vitro but also to the suppression of IL-6 secretion in vivo. Lastly, we provide evidence that immune escape by PGN *O*-acetylation is critical for *Listeria* virulence.

METHODS

Bacterial Strains, Growth Conditions, and Cell Wall Preparation

L. monocytogenes EGDe (serovar 1/2a, BUG1600) was used as the parental strain [23]. *L. monocytogenes* Δ*pgdA* (BUG2288), *L. monocytogenes Δhly* (BUG2133), and *L. monocytogenes* Δ*oatA* (BUG2410) were obtained by gene deletion from EGDe, and *L. monocytogenes* Δ*oatA*Δ*pgdA* (BUG2519) was obtained by gene deletion from Δ*pgdA* as described [24]. *L. monocytogenes* Δ*oatA* complemented strain (BUG2520) was obtained from Δ*oatA* as described [25]. Oligonucleotides used for gene deletion and complementation are listed in Supplementary Table S1; online only. *Listeria* strains were grown in brain-heart infusion (BHI) broth (BD) at 37°C and 200 rpm. Highly purified cell wall from *L. monocytogenes* strains was prepared as described [20].

Cells, Culture Media, and Cell Infection

Murine macrophage-like cell line RAW264.7 (ATCC:TIB-71) was cultured in Dulbecco's modified Eagle medium (Gibco) containing 10% decomplemented fetal bovine serum (Biowest) at 37°C in 10% CO_2 atmosphere. Peritoneal-elicited macrophages (PEM) and bone marrow–derived macrophages (BMDM) isolated from 8-week-old female C57BL/6J mice (Charles River Laboratories) as described [20, 26] and human acute monocytic leukemia cell line THP-1 (ATCC:TIB-202) were cultured in RPMI-1640 (Gibco) containing 10% decomplemented fetal bovine serum (Biowest) at 37°C in 10% CO_2 atmosphere. PEM and BMDM cells were infected with *L. monocytogenes* at multiplicity of infection (MOI) 10:1 and 20:1, respectively, centrifuged at 300*g* for 2 minutes, and incubated at 37°C for 15 minutes to allow bacterial phagocytosis. RAW264.7 and THP-1 cells were infected with *L. monocytogenes* at MOI 25:1 and 10:1, respectively, over a period of 1 hour at 37°C. The number of intracellular bacteria was assessed at various time points, and infected RAW264.7 cells were observed by electron microscopy as described [20]. Experiments were repeated 2 to 3 times independently.

Antibacterial Activity Assays

Lysozyme antibacterial activity was assessed by the disk diffusion method. Overnight cultures of *Listeria* strains were diluted in BHI broth to 10^6 colony-forming unit (CFU)/mL and spread on BHI agar plates. Disks (10 mm) were loaded with 10 μL of chicken egg white lysozyme (Sigma). Plates were incubated for 48 hours at 37°C and inhibition zone diameter was measured. Cefotaxime minimum inhibitory concentration (MIC) was determined using E-test strips (Biomérieux) on BHI agar plates that were inoculated with *Listeria* strains. Inhibitory activity of antimicrobial peptides was determined in 96-well plates. Overnight *Listeria* cultures were diluted in BHI broth to 10^6 CFU/mL and incubated with antimicrobial peptides at 37°C and 200 rpm overnight. CFUs in each well were assessed by plating serial dilutions on BHI agar.

Virulence Studies and Cytokine Production in Mice

Experiments were performed according to the Institut Pasteur guidelines for animal experimentation. Median lethal dose (LD_{50}), mice survival, and quantification of bacterial multiplication were carried out in BALB/c mice following intravenous challenge or in *iFABP-hEcad* transgenic mice after oral infection as described [20]. Cytokine concentrations in infected mice were measured by the Mouse cytokine 20-plex bead immunoassay (Biosource) and the Mouse Interferon-Beta enzyme-linked immunosorbent assay (ELISA; PBL).

Statistical Analysis

Results are expressed as mean values ± SD of 3–8 samples. Student *t* test was performed to determine statistical significance (*, **, and *** indicate $P < .05$, $P < .01$, and $P < .001$, respectively).

RESULTS

The *lmo1291* Gene Encodes a Peptidoglycan *O*-Acetyltransferase

The genome of *L. monocytogenes* EGDe encodes a putative *O*-acetyltransferase, Lmo1291, with a calculated molecular mass of 70 423 Da and an estimated isoelectric point (pI) of 10.1. The *lmo1291* gene is surrounded by *lmo1290* and *lmo1292,* which encode the internalin-like protein InlK (27) and a putative phosphodiesterase, respectively, and are both transcribed divergently compared with *lmo1291* (Figure 1*A*). While *lmo1290* is absent from the *L innocua* genome, *lmo1291* orthologs are present in *Listeria* species whose genome has been sequenced, including *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. The 622-amino-acid protein encoded by *lmo1291* contains a signal peptide followed by an acyltransferase domain (COG1835) and a YrhL-like hydrolase domain (CD01840), which belongs to the superfamily of SGNH hydrolases (Figure 1*B*). Orthologs of Lmo1291 are present in *Staphylococcus aureus* [28] and other bacteria such as *Enterococcus faecalis* and *Lactococcus lactis*. Alignment of amino-acid sequences from Lmo1291 orthologs shows that the Ser/Asp/His catalytic triad found in SGNH hydrolases is conserved (Figure 1*C*). Lmo1291 and its orthologs contain the same domains and several putative transmembrane regions, suggesting that these enzymes are associated with the cell wall.

In order to assess the function of Lmo1291, the *lmo1291* gene of *L. monocytogenes* EGDe was deleted by allelic exchange. Purified cell wall was prepared from EGDe and Δ*lmo1291* strains and hydrolyzed by mutanolysin to generate muropeptides. Muropeptides were separated by high-pressure liquid chromatography (HPLC) and their composition was determined by mass spectrometry (Figure 1*D*). Analysis of each peak revealed that EGDe has *O*-acetylated *N*-acetyl muramic acid residues. Approximately 23% of *L. monocytogenes* muropeptides contained *O*-acetylated residues (Supplemental Table S2; online only). The Δ*lmo1291* mutant lacked these peaks. Reduction of EGDe muropeptides at pH 9, a treatment that removes *O*-acetylation, resulted in disappearance of the muropeptides that were found only in EGDe and not in Δ*lmo1291* (data not shown). Thus, these muropeptides were all modified by *O*-acetylation. These results indicate that *lmo1291*, renamed *oatA*, encodes a PGN *O*-acetyltransferase (Figure 1*E*).

OatA Confers Resistance to Antimicrobial Compounds

As we previously demonstrated, *Listeria* PGN *N*-deacetylase was a major determinant of lysozyme resistance [20]. We then tested whether OatA could contribute to lysozyme resistance. EGDe and Δ*oatA* growth rates were similar in BHI (data not shown). Growth of Δ*oatA* was significantly inhibited by lysozyme as shown by a disk diffusion assay, in contrast to EGDe (Figure 2*A*). To demonstrate that lysozyme sensitivity was directly due to absence of PGN *O*-acetylation, Δ*oatA* was complemented by chromosomal insertion of a single copy of *oatA* and its promoter. Complementation fully restored lysozyme resistance (Figure 2*A*). To determine if the OatA protective role was specific to lysozyme, we tested Δ*oatA* susceptibility to other compounds targeting cell wall such as the β-lactam antibiotic cefotaxime and the *Staphylococcus gallinarum* lantibiotic gallidermin. Cefotaxime MIC for Δ*oatA* was 8-fold lower than that of EGDe, which was similar to that of Δ*oatA*+*oatA* (Figure 2*B*). Addition of increasing concentrations of gallidermin to cultures correlated with decreasing survival of Δ*oatA* compared with EGDe (Figure 2*C*). Hence, OatA protection is not specific to lysozyme and extends to other antimicrobial compounds.

OatA Contributes to Listeria Resistance to Macrophage Antimicrobial Activity

Since killing mechanisms of macrophages include production of lysozyme and other antimicrobial compounds, we assessed OatA contribution to the intracellular survival of *Listeria*. While EGDe could replicate in THP-1 cells from 30 minutes to 4 hours post infection, Δ*oatA* growth was controlled (Figure 2*D*). Furthermore, Δ*oatA* was killed faster and more efficiently than EGDe in PEM (Figure 2*E*) and BMDM (Figure 2*F*), as there were 10 times fewer Δ*oatA* than EGDe 24 hours post infection. The ability of Δ*oatA* to multiply in RAW264.7 cells was also impaired after 2 hours of infection compared with EGDe and Δ*oatA*+*oatA* complemented strain (Figure 2*G*). Next, we observed infected RAW264.7 cells by electron microscopy. In contrast to Δ*pgdA* [20], Δ*oatA* did not accumulate in vacuoles and was readily observed in the cytosol (Figure 2*H*). However, in contrast to EGDe, Δ*oatA* was apparently not forming protrusions (Figure 2*H*). While similar to the number of EGDe per cell 30 minutes post infection, the number of Δ*oatA* was lower than that of EGDe after 4 hours (Figure 2*I*). These results indicate that OatA contributes to survival within macrophages, possibly by protecting *Listeria* from killing mechanisms, such as lysis mediated by antimicrobial polypeptides.

OatA Is Required for Pathogenesis

Because antimicrobial polypeptides and macrophages are major components of innate immunity, we next addressed the role of OatA in vivo. We first determined Δ*oatA* LD_{50} in BALB/c mice infected intravenously. Virulence of Δ*oatA* was severely attenuated, as values of LD_{50} were 1.1×10^7 and 0.7×10^3 bacteria for Δ*oatA* and EGDe, respectively. Intravenous injection of 10^6 EGDe resulted in 100% mortality in 3 days (Figure 3*A*). In contrast, mice infected with 10^6 Δ*oatA* survived (Figure 3*A*). Next, we analyzed the survival of Δ*oatA* in BALB/c mice after intravenous injection of a sublethal infectious dose. Δ*oatA* had a reduced capacity to replicate in liver and spleen as early as 6 hours post infection, while EGDe readily colonized both organs (Figure 3*B*). At 48 hours post infection, liver and spleen colonization by Δ*oatA* was reduced 100 times and 1000 times, respectively, compared with EGDe. Bacteremia could not be

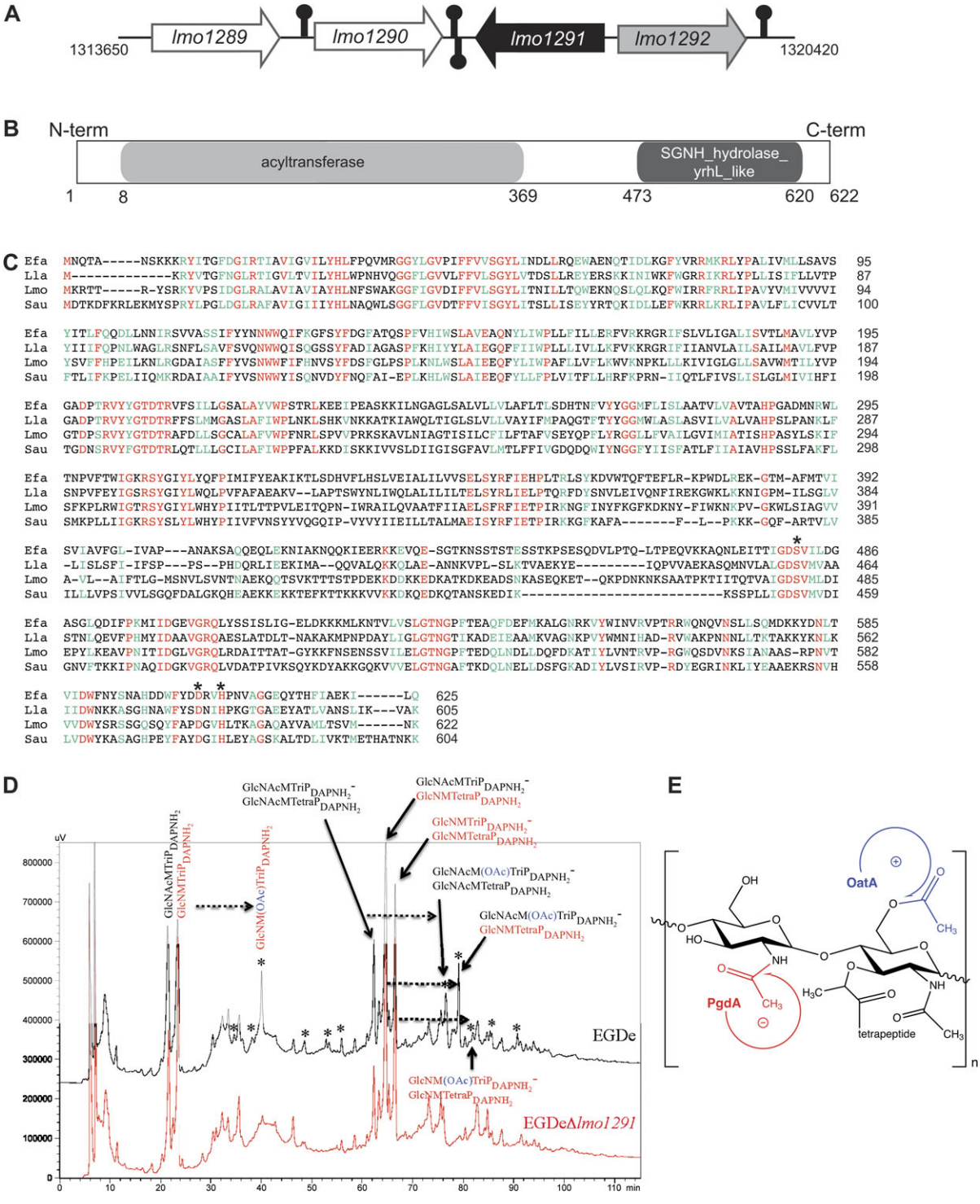

Figure 1. Lmo1291 is a peptidoglycan *O*-acetyltransferase. *A*, Genetic organization of the *L monocytogenes lmo1291* gene locus (in black) showing chromosomal coordinates (bp), putative terminators (black hairpins), and the flanking genes *lmo1290* encoding a putative internalin (in white) and *lmo1292* encoding a putative glycerophosphodiester phosphodiesterase (in gray). *B*, Map of putative domains of Lmo1291 (amino acid). *C*, Sequence alignment of *O*-acetyltransferases from *Enterococcus faecalis* (Efa), *Lactococcus lactis* (Lla), *Staphylococcus aureus* (Sau), and *L. monocytogenes* Lmo1291 (Lmo). Sequences were aligned using T-Coffee software. Amino acids in red are identical and residues in green are similar. Asterisks indicate the conserved catalytic triad of SGNH hydrolases. Efa, Lla, and Sau orthologs show 56%, 50%, and 55% identity to Lmo1291, respectively. *D*, Muropeptide profile of the highly purified cell walls of EGDe and its isogenic Δ*oatA* mutant. Peaks highlighted by an asterisk indicate muropeptides absent from the Δ*oatA* mutant. Major peaks were analyzed by MALDI MS/MS and their respective structure are indicated above the corresponding peak. Deacetylated muropeptides are indicated in red. Muropeptides that are *O*-acetylated are highlighted by the addition of the OAc moiety in blue. Dotted arrows indicate the relationship between the different

detected at 24 hours following infection with Δ*oatA* (Figure 3*B*). At 48 hours post infection, inactivation of *oatA* decreased bacteremia by 2 $\log_{10}$. Thus, OatA is a novel major virulence determinant that contributes to early stages of listeriosis in mice.

The oral route is the natural way of infection by *Listeria* and allows for the study of early stages of infection, including survival of bacteria in the intestinal lumen. Transgenic mice expressing human E-cadherin, which are permissive to *Listeria* oral infection, were thus infected intragastrically with EGDe or Δ*oatA*. In the intestinal lumen, the number of Δ*oatA* bacteria was strongly decreased 24 hours postinfection (Figure 3*C*). There were also significantly fewer mutant than wild-type bacteria in small intestine tissue (Figure 3*C*). Hence, Δ*oatA* is deficient in very early stages of infection in the intestinal lumen and at later stages of colonization in intestinal tissue.

OatA Decreases Cytokine Production in Infected Mice

We previously demonstrated that PGN *N*-deacetylation reduces *Listeria* capacity to induce secretion of inflammatory cytokines by macrophages in vitro [20]. We hypothesized that PGN *O*-acetylation also affects host cytokine response in vivo. We thus studied Δ*oatA* capacity to induce cytokine production in the liver, a major site of *Listeria* replication. We first infected BALB/c mice intravenously with a sublethal dose of EGDe or Δ*oatA*. Production of 21 cytokines was then determined by immunoassay. In mice infected with EGDe, levels of most cytokines tested increased in correlation with an increased number of bacteria (data not shown). Δ*oatA* induced higher levels of 7 cytokines compared with EGDe 6 hours post infection (Figure 3*D*). Strikingly, production of the inflammatory cytokine IL-6 in response to EGDe reached only 60% of that induced by Δ*oatA* both at early (6 hours) and late (72 hours) time points of infection. At 6 hours post infection, the production of T-helper 1 (Th1) differentiation cytokine IL-12, proinflammatory cytokine interleukin 2 (IL-2), chemokine monocyte chemoattractant protein 1 (MCP-1), monokine induced by IFN-γ (MIG) and macrophage inflammatory protein-1α (MIP-1α), and T-helper 2 (Th2) cytokine interleukin 5 (IL-5) increased more in response to Δ*oatA* compared with EGDe (Figure 3*D*), while liver colonization by Δ*oatA* was already lower than that of EGDe (Figure 3*B*). At later time points, the cytokine production in response to Δ*oatA* was similar (IL-2, MIG, IL-5) or even lower (IL-12, MCP-1, MIP-1α) compared with that induced by EGDe (Figure 3*D*). Interestingly, cytokine production in response to infection with the Δ*hly* mutant was severely reduced compared with that induced by EGDe (Supplemental Figure S1*A*). This mutant, which does not produce LLO, was cleared from mice more rapidly than Δ*oatA* (Supplemental Figure S1*B*). Consequently, it did not activate any cytokine response in contrast to Δ*oatA*. Overall, OatA controls the production of inflammatory mediators at early stages of *Listeria* infection.

Peptidoglycan *O*-Acetylation and *N*-Deacetylation Control Different Cytokine Responses In Vivo

In order to test whether *O*-acetylation led to a specific cytokine profile or if another PGN modification could control production of the same cytokines, 21 cytokines were quantitated in the liver of mice infected with Δ*pgdA*, which does not produce PGN deacetylase. BALB/c mice were first injected intravenously with a sublethal dose of EGDe or Δ*pgdA*. Bacterial growth and cytokine production were determined in the liver at 6, 24, 48, and 72 hours after infection. Colonization of liver and spleen by Δ*pgdA* was strongly impaired compared with EGDe (Figure 4*A*). At 48 hours post infection, the number of Δ*pgdA* bacteria decreased by 3 and 2 $\log_{10}$ compared with EGDe in the liver and spleen, respectively. At 72 hours post infection with Δ*pgdA*, colonization decreased by 4 and 2 $\log_{10}$ in the liver and spleen, respectively. In contrast to EGDe, Δ*pgdA* could not be detected in the bloodstream (Figure 4*A*).

Unlike Δ*oatA*, Δ*pgdA* did not induce IL-6 in the liver of infected mice (Figure 4*B*). Production of IL-6 in response to Δ*pgdA* was similar to that of EGDe until 48 hours post infection and lower at 72 hours. At 6 hours post infection, production of IL-12, IL-2, MCP-1, MIG, MIP-1α, and IL-5 was similar in the liver of mice infected by Δ*pgdA* and EGDe (Figure 4*B*). While strongly attenuated in its capacity to colonize the liver and spleen, as Δ*pgdA*, Δ*oatA* stimulates the production of a specific subset of cytokines, including IL-6. Thus, PGN *O*-acetylation and *N*-deacetylation represent 2 nonredundant modifications allowing *Listeria* to escape from innate immune response.

We hypothesized that the nonredundant functions of OatA and PgdA in immune escape could synergize and increase the capacity of *Listeria* to colonize the host. We thus created a strain of *L. monocytogenes* unable to produce OatA and PgdA and studied its virulence in vivo. BALB/c mice were infected intravenously with a sublethal dose of EGDe or Δ*pgdA*Δ*oatA*. Growth of the double mutant was strongly impaired compared with that of EGDe in the liver and spleen (Figure 4*C*). Mutant bacteria could not be detected in the liver and spleen 24 hours post infection, while EGDe efficiently colonized both organs and single mutants had a defective but detectable capacity to colonize these organs (Figures 3*B* and 4*A*, 4*C*). At 48 hours post infection, Δ*pgdA*Δ*oatA* was undetectable in the spleen and poorly colonized the liver compared with EGDe. At 72 hours post infection, there were $7-\log_{10}$ and $5-\log_{10}$ differences between the number of Δ*pgdA*Δ*oatA* and EGDe in the liver and spleen, respectively.

O-acetylated muropeptides and their parent muropeptides that lack the OAc moiety. *E*, Localization of PGN modifications by OatA (*O*-acetylation in blue) and PgdA (*N*-deacetylation in red). MALDI indicates matrix-assisted laser desorption ionization; MS/MS, tandem mass spectrometry.

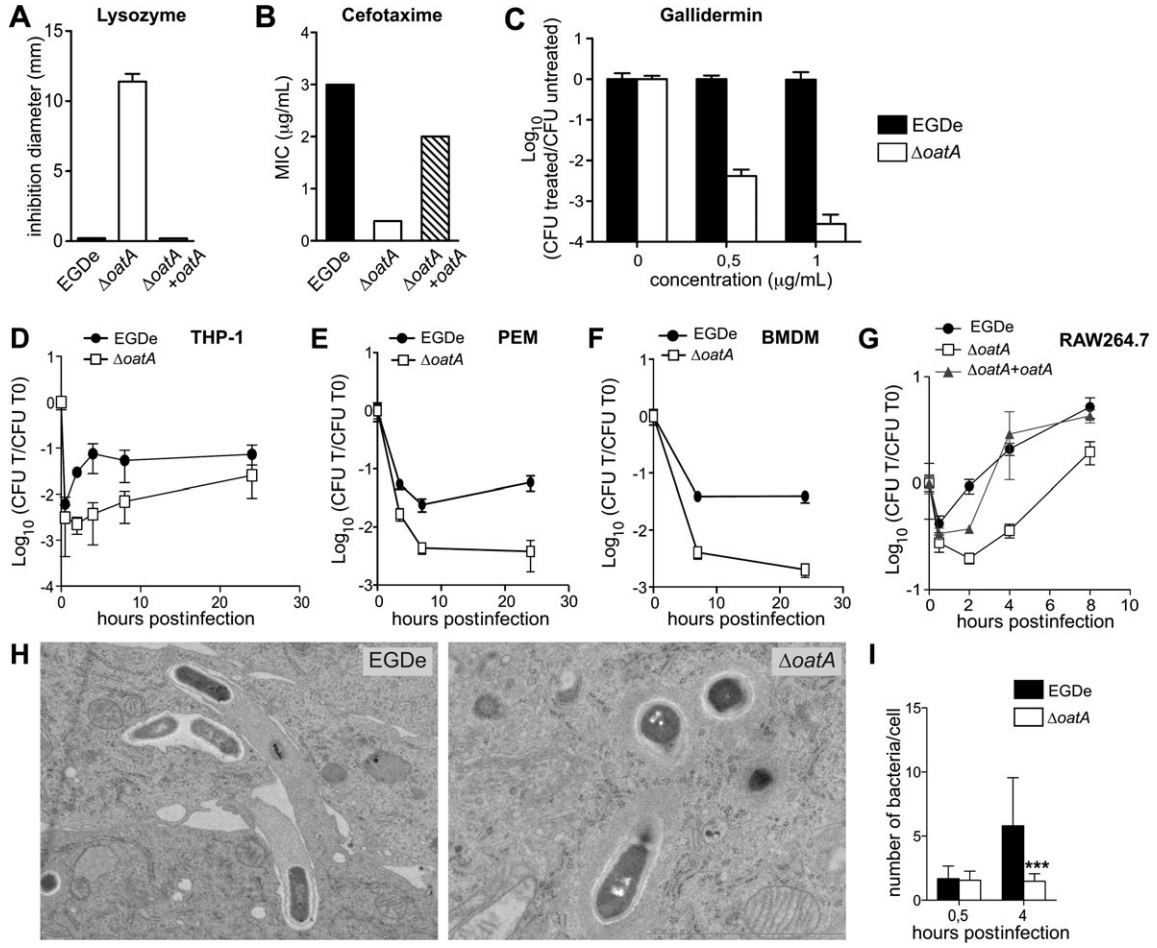

Figure 2. Inactivation of *oatA* increases sensitivity to antimicrobial molecules that target bacterial cell wall and impairs *L monocytogenes* survival in macrophages. *A*, Lysozyme disk-diffusion assay. Growth inhibition caused by lysozyme loaded on a paper disk (1 mg/disk) was measured on BHI agar plates inoculated with EGDe (black bars), the Δ*oatA* mutant (white bars), or Δ*oatA*+ *oatA*−complemented strains (hatched bars). *B*, Cefotaxime minimum inhibitory concentration was determined using E-test strips on BHI agar plates that were inoculated with EGDe (black bars), Δ*oatA* mutant (white bars), or Δ*oatA*+ *oatA*−complemented strains (hatched bars). *C*, Gallidermin inhibitory activity was determined in 96-well plates. 10^6 CFU/mL of EGDe (black bars) or Δ*oatA* (white bars) were incubated with increasing concentrations of gallidermin. The number of CFU in each well was assessed after overnight incubation at 37°C by plating serial dilutions on BHI agar plates. *D*, THP-1 cells ($n = 4$); *E*, peritoneal-elicited macrophages ($n = 5$); and *F*, bone marrow–derived macrophages ($n = 4$) were infected with EGDe (black circles) or Δ*oatA* (white squares). *G*, RAW264.7 cells ($n = 5$) were infected with EGDe (black circles), Δ*oatA* (white squares), or the Δ*oatA*+ *oatA* complemented strain (gray triangles). The number of CFU was determined at different time points after cell lysis with 0.2% triton. Data are means ± SD. (*H–I*) Electron microscopy analysis of RAW264.7 cells infected with EGDe or Δ*oatA*. (*H*) RAW264.7 cells after 4 hours of infection with EGDe or Δ*oatA*. Left panel: EGDe in vacuoles and protrusions; right panel: Δ*oatA* free in the cytosol. Scale bars: 2 μm. *I*, The number of bacteria per cell was determined by counting intravacuolar and cytosolic bacteria 30 minutes and 4 hours postinfection. Data are means ± SD ($n = 25$). Student *t* test was performed to determine statistical significance (*** indicates $P < .001$). CFU, colony-forming unit.

Furthermore, Δ*pgdA*Δ*oatA* could not be detected in the bloodstream (Figure 4*C*). Overall, the virulence of the double mutant is more attenuated than that of single mutants. Thus, OatA and PgdA are nonredundant virulence determinants of *L. monocytogenes* that contribute to listeriosis in mice.

DISCUSSION

The composition of the *L. monocytogenes* cell wall and its role in virulence remain to be fully characterized. Here, we report that *Listeria O*-acetylates its PGN and that this modification is critical for virulence and escape from innate immune response. Analysis of the *L. monocytogenes* EGDe genome revealed a gene encoding a putative membrane-bound *O*-acetyltransferase encoded by the gene *lmo1291*. Comparison of PGN from EGDe and an *lmo1291* deletion mutant confirmed that this gene, which we renamed *oatA*, encodes a PGN *O*-acetyltransferase. This is the second enzyme involved in *Listeria* PGN modification after PgdA, an enzyme that deacetylates *N*-acetylglucosamine residues [20].

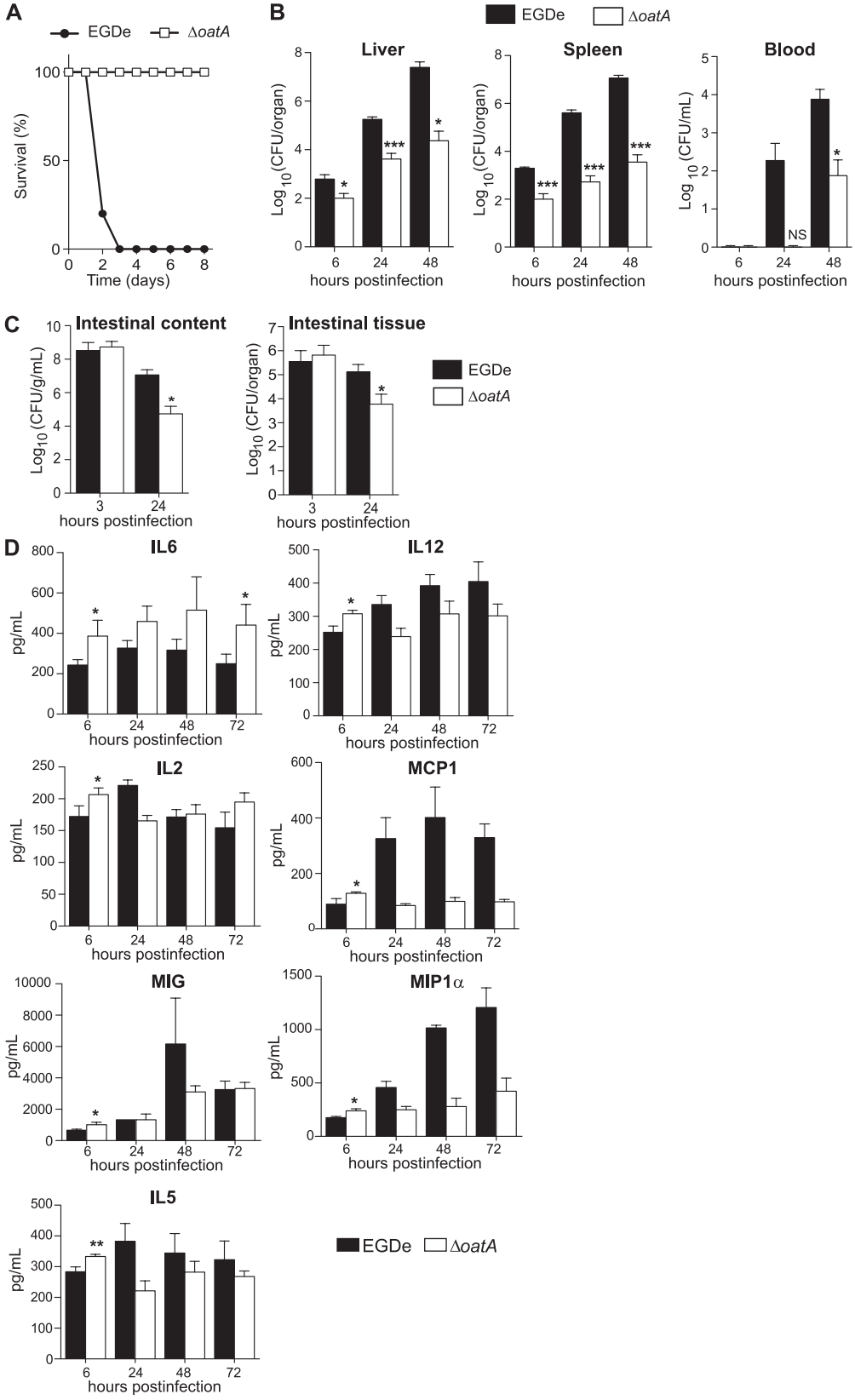

Figure 3. The Δ*oatA* mutant has a strongly attenuated virulence in mice and triggers an increased cytokine response early after infection. *A*, BALB/c mice were challenged by intravenous injection of 10^6 EGDe (black circles) or Δ*oatA* (white squares). Survival of infected mice was determined over time ($n = 5$). *B*, BALB/c mice were challenged by intravenous injection of a sublethal dose (10^4 bacteria per mouse) of EGDe (black bars) or Δ*oatA* (white bars).

Inactivation of *oatA* increased *L. monocytogenes*' sensitivity to lysozyme, a major component of the innate defense that hydrolyzes PGN between *N*-acetylglucosamine and *N*-acetylmuramic acid residues [22]. We reported that PgdA confers resistance to lysozyme [20]. Interestingly, the Δ*pgdA* mutant is more sensitive to lysozyme than the Δ*oatA* mutant because it is killed by 10 μg/mL of lysozyme [20], a concentration that did not alter Δ*oatA* viability. Importantly, the OatA protective role was not specific to lysozyme because Δ*oatA* was more sensitive to cefotaxime and gallidermin, antibacterial compounds targeting the cell wall. Since Δ*pgdA* is not more sensitive to cefotaxime (data not shown), our results suggest that fine structural modifications of muropeptides change the sensitivity to different inducers of bacterial cell lysis and that the 2 modifications are not redundant.

Inactivation of *oatA* strongly reduced *Listeria* capacity to replicate in THP-1 and RAW264.7 cells and to survive in murine PEM and BMDM. In contrast, the mutant was not affected in its capacity to enter and survive in epithelial cells (data not shown). Inactivation of *pgdA* also severely impairs the ability of *Listeria* to survive and multiply in macrophages but not in epithelial cells [20]. However, while Δ*pgdA* accumulates in vacuoles after phagocytosis [20], Δ*oatA* does not. PGN modifications by OatA and PgdA thus protect *Listeria* from killing by phagocytes through nonredundant mechanisms, possibly by conferring different levels of resistance to cell wall–targeting molecules such as antimicrobial peptides and lysozyme or by conferring resistance to different spectra of host antimicrobial molecules.

OatA is critical for efficient host colonization by *Listeria*. Virulence of Δ*oatA* is highly attenuated in mice. The LD_{50} of Δ*oatA* was comparable to that of an *hly* mutant, one of the *Listeria* mutants whose virulence is the most attenuated [29]. OatA plays a role both at early and later stages of murine listeriosis, as indicated by host control of Δ*oatA* in intestinal lumen and tissue, the bloodstream, liver, and spleen. The other PGN modification, *N*-deacetylation, is also required for *Listeria* survival at early stages of infection and after crossing the intestinal barrier [20]. Thus, in *Listeria*, PGN modification enzymes are among the most important virulence factors.

Inactivation of OatA in *L. monocytogenes* amplified the magnitude of inflammatory cytokine and chemokine responses, but not interferon-β response, early after infection and, in the case of IL-6, at later stages of infection. Infection by Δ*oatA* and Δ*pgdA* led to different cytokine responses. Thus, OatA and PgdA have nonredundant roles in dampening inflammatory cytokine production. Several other *L. monocytogenes* virulence factors interfere with cytokine production. InlH, a cell-surface protein of the internalin family, controls IL-6 production during murine listeriosis by an unknown mechanism [30]. We recently reported that InlC, a secreted internalin, dampens production of MIP-1α and IL-8 homolog KC in infected mice by targeting the α subunit of IκB kinase [31]. These studies demonstrate that, as in other pathogenic bacteria, *L. monocytogenes* evades innate immune response through synthesis of multiple virulence determinants.

The *oatA* gene has been studied in *S. aureus* [28] and was shown to have a minor, although significant, role in bacterial survival in a murine model of skin infection [32]. Interestingly, *S. aureus* Δ*oatA* induces IL-1β secretion by infected macrophages in vitro and triggers an IL-1β- and IL-18-dependent skin inflammation after subcutaneous infection of mice [32]. *L. monocytogenes* Δ*oatA* did not increase IL-1β production in our murine model of infection. Thus, the magnitude of virulence defect of Δ*oatA* mutants and the spectrum of cytokine induced in response to infection with Δ*oatA* mutants vary between pathogens.

In conclusion, we have identified a new modification of *L. monocytogenes* PGN that is critical for infection. This modification relies on the *O*-acetyltransferase OatA and confers resistance to antimicrobial compounds, including lysozyme. OatA is required for intracellular survival of *L. monocytogenes* in macrophages and for the efficient control of cytokine response in vivo. Our study reveals that PGN *O*-acetylation is a novel mechanism used by *Listeria* to evade innate immunity and ultimately colonize the host successfully. It reinforces the notion that PGN is a pivotal component in microbe–host interactions.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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Colonization of liver, spleen, and blood was followed 6, 24, and 48 hours postinfection. Data are means ± SD ($n = 4$). *C*, Transgenic human E-cadherin mice were used as a permissive model for the oral route of infection. These mice were infected with 10^{10} EGDe (black bars) or Δ*oatA* (white bars). After 3 hours and 24 hours, mice were euthanized and the number of bacteria in the intestinal lumen and intestinal tissue was determined. Data are means ± SD ($n = 3$–5). *D*, BALB/c mice were inoculated intravenously with 5×10^3 bacteria. The liver was dissected and homogenized. Homogenates were assayed using a multiplex immunoassay to determine cytokine level in response to infection with EGDe (black bars) or Δ*oatA* (white bars). Data are means ± SD ($n = 4$). Student *t* test was performed to determine statistical significance: *, **, and *** indicate $P < .05$, $P < .01$, and $P < .001$, respectively.

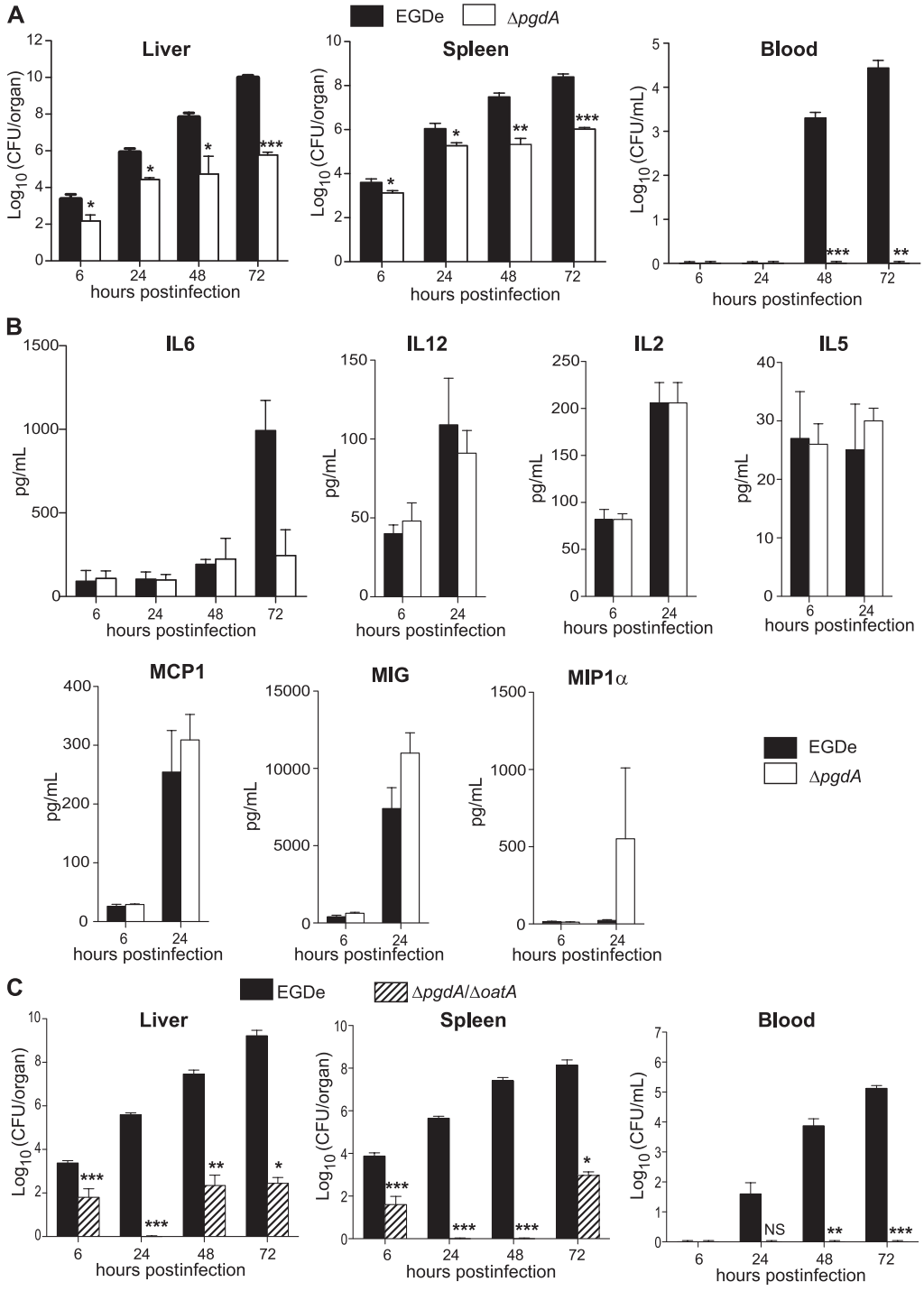

Figure 4. The Δ*pgdA* mutant, while highly attenuated in virulence, does not induce IL6 and is more virulent than a Δ*pgdA*Δ*oatA* double mutant. *A*, BALB/c mice were challenged by intravenous injection of a sublethal dose (10^4 bacteria per mouse) of the parental EGDe strain (black bars) and its isogenic Δ*pgdA* mutant (white bars). Colonization of liver, spleen, and blood was followed 6, 24, 48, and 72 hours postinfection. Data are means ± SD ($n = 4$). *B*, BALB/c mice were inoculated intravenously with 5×10^3 bacteria. The liver was dissected and homogenized. Homogenates were assayed to determine cytokine level in response to infection with the parental EGDe strain (black bars) or its isogenic Δ*pgdA* mutant (white bars). Data are means ± SD ($n = 4$). *C*, BALB/c mice were challenged by intravenous injection of a sublethal dose (10^4 bacteria per mouse) of the parental EGDe strain (black bars) and its isogenic Δ*pgdA*Δ*oatA* mutant (hatched bars). Colonization of liver, spleen, and blood was followed at 6, 24, 48, and 72 hours postinfection. Data are means ± SD ($n = 4$). Student *t* test was performed to determine statistical significance: *, **, and *** indicate $P < .05$, $P < .01$, and $P < .001$, respectively.

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References

1. Vazquez-Boland JA, Kuhn M, Berche P, et al. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev **2001**; 14: 584–640.
2. Pizarro-Cerda J, Cossart P. *Listeria monocytogenes* membrane trafficking and lifestyle: the exception or the rule? Annu Rev Cell Dev Biol **2009**; 25:649–70.
3. Hamon M, Bierne H, Cossart P. *Listeria monocytogenes*: a multifaceted model. Nat Rev Microbiol **2006**; 4:423–34.
4. Pamer EG. Immune responses to *Listeria monocytogenes*. Nat Rev Immunol **2004**; 4:812–23.
5. Stavru F, Archambaud C, Cossart P. Cell biology and immunology of *Listeria monocytogenes* infections: novel insights. Immunol Rev **2011**; 240:160–84.
6. Conlan JW, North RJ. Neutrophils are essential for early anti-*Listeria* defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. J Exp Med **1994**; 179:259–68.
7. Rogers HW, Unanue ER. Neutrophils are involved in acute, nonspecific resistance to *Listeria monocytogenes* in mice. Infect Immun **1993**; 61:5090–6.
8. Mocci S, Dalrymple SA, Nishinakamura R, Murray R. The cytokine stew and innate resistance to *L. monocytogenes*. Immunol Rev **1997**; 158:107–14.
9. Dalrymple SA, Lucian LA, Slattery R, et al. Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. Infect Immun **1995**; 63:2262–8.
10. Kopf M, Baumann H, Freer G, et al. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature **1994**; 368:339–42.
11. Tripp CS, Wolf SF, Unanue ER. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc Natl Acad Sci USA **1993**; 90:3725–9.
12. Dai WJ, Bartens W, Kohler G, Hufnagel M, Kopf M, Brombacher F. Impaired macrophage listericidal and cytokine activities are responsible for the rapid death of *Listeria monocytogenes*–infected IFN-gamma receptor-deficient mice. J Immunol **1997**; 158:5297–304.
13. Havell EA. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J Immunol **1989**; 143:2894–9.
14. Kato K, Nakane A, Minagawa T, et al. Human tumor necrosis factor increases the resistance against *Listeria* infection in mice. Med Microbiol Immunol **1989**; 178:337–46.
15. Nakane A, Minagawa T, Kohanawa M, et al. Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infections. Infect Immun **1989**; 57:3331–7.
16. Pfeffer K, Matsuyama T, Kundig TM, et al. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. Cell **1993**; 73:457–67.
17. Rothe J, Lesslauer W, Lotscher H, et al. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. Nature **1993**; 364: 798–802.
18. Harty JT, Bevan MJ. Specific immunity to *Listeria monocytogenes* in the absence of IFN gamma. Immunity **1995**; 3:109–17.
19. Diacovich L, Gorvel JP. Bacterial manipulation of innate immunity to promote infection. Nat Rev Microbiol **2010**; 8:117–28.
20. Boneca IG, Dussurget O, Cabanes D, et al. A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. Proc Natl Acad Sci USA **2007**; 104:997–1002.
21. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature **2006**; 442:39–44.
22. Callewaert L, Michiels CW. Lysozymes in the animal kingdom. J Biosci **2010**; 35:127–60.
23. Glaser P, Frangeul L, Buchrieser C, et al. Comparative genomics of *Listeria* species. Science **2001**; 294:849–52.
24. Archambaud C, Gouin E, Pizarro-Cerda J, Cossart P, Dussurget O. Translation elongation factor EF-Tu is a target for Stp, a serine-threonine phosphatase involved in virulence of *Listeria monocytogenes*. Mol Microbiol **2005**; 56:383–96.
25. Archambaud C, Nahori MA, Pizarro-Cerda J, Cossart P, Dussurget O. Control of *Listeria* superoxide dismutase by phosphorylation. J Biol Chem **2006**; 281:31812–22.
26. Alford CE, King TE Jr, Campbell PA. Role of transferrin, transferrin receptors, and iron in macrophage listericidal activity. J Exp Med **1991**; 174:459–66.
27. Dortet L, Mostowi S, Samba Louaka A, et al. Recruitment of the major vault protein by InlK: a Listeria monocytogenes strategy to avoid autophagy. PLoS Pathogens. In press.
28. Bera A, Herbert S, Jakob A, Vollmer W, Gotz F. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol Microbiol **2005**; 55:778–87.
29. Portnoy DA, Jacks PS, Hinrichs DJ. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J Exp Med **1988**; 167: 1459–71.
30. Personnic N, Bruck S, Nahori MA, et al. The stress-induced virulence protein InlH controls interleukin-6 production during murine listeriosis. Infect Immun **2010**; 78:1979–89.
31. Gouin E, Adib-Conquy M, Balestrino D, et al. The *Listeria monocytogenes* InlC protein interferes with innate immune responses by targeting the IκB kinase subunit IKKα. Proc Natl Acad Sci USA **2010**; 107:17333–8.
32. Shimada T, Park BG, Wolf AJ, et al. *Staphylococcus aureus* evades lysozyme-based peptidoglycan digestion that links phagocytosis, inflammasome activation, and IL-1beta secretion. Cell Host Microbe **2010**; 7:38–49.