Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins

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Toll-like receptors and other immune-signaling pathways play important roles as sensors of bacterial pattern molecules, such as peptidoglycan, lipoprotein, or teichoic acid, triggering innate host immune responses that prevent infection. Immune recognition of multiple bacterial products has been viewed as a safeguard against stealth infections; however, this hypothesis has never been tested for *Staphylococcus aureus*, a frequent human pathogen. By generating mutations that block the diacylglycerol modification of lipoprotein precursors, we show here that *S. aureus* variants lacking lipoproteins escape immune recognition and cause lethal infections with disseminated abscess formation, failing to elicit an adequate host response. Thus, lipoproteins appear to play distinct, nonredundant roles in pathogen recognition and host innate defense mechanisms against *S. aureus* infections.

innate immunity | virulence

To establish a focus of infection and resultant bacteremia, *Staphylococcus aureus* overcomes physical protective barriers of the human body through invasion of soft tissues, surgical wounds, or medical devices (1). In the first hours after infection, staphylococci are cleared from the blood stream, in part by way of phagocytic killing but also by bacterial binding to host organ tissues (2). Staphylococci that escape killing replicate in infected tissues and generate proinflammatory responses mediated by the release of cytokines and chemokines from macrophages, neutrophils, and other immune cells (3, 4). The resulting massive invasion of immune cells to the site of infection is accompanied by central liquefaction necrosis and formation of peripheral fibrin walls in an effort to prevent microbial spread and allow for removal of necrotic tissue debris (5). When launched early and effectively, innate immune responses limit the establishment of infectious foci and thereby curb the severity of staphylococcal infections. These early events culminate in the activation of adaptive immune responses, during which T and B cells capable of specific antigen recognition lead to the eradication of staphylococci. Thus, the coordinated action of the innate and adaptive immune response is critical for efficient pathogen elimination.

Binding of bacterial molecules called pathogen-associated molecule patterns (PAMPs) to dedicated Toll-like receptors (TLRs) or Nod proteins triggers specific signaling events and host responses to invading pathogens (6, 7). To date, a dozen different TLRs have been identified in mammals (8). TLR2 plays a critical role in host defense against *S. aureus*, because TLR2 knockout mice are highly susceptible to i.v. infection with staphylococci (9). Purified staphylococcal PAMPs activate immune signaling through TLR2 both *in vivo* and in cell culture (10–12) (Fig. 1*A*). Furthermore, bacterial lipoproteins also function as PAMPs, activating TLR2 signaling cascades (12–15). However, the contribution of individual PAMPs to host recognition of invading staphylococci by immune surveillance systems has not been studied.

By generating mutations that block diacylglycerol attachment to lipoprotein precursors, we show that *S. aureus* variants bearing apolipoproteins escape immune recognition and cause disseminated abscess formation with increased lethality during infection. Furthermore, we show that immune cells do not infiltrate sites of infection carrying these mutant bacteria. Hence, it appears that acylation of lipoproteins is required for initiating and sustaining effective immune responses from infected hosts. Understanding the role of bacterial lipoproteins in mediating innate and adaptive immunity will be useful for the therapy of human *S. aureus* infections.

Results

Genetic Requirement of Staphylococcal PAMPs. To examine the contribution of staphylococcal PAMPs to immune recognition and disease pathogenesis, a collection of *S. aureus bursa aurealis* mutants (Phoenix library) (16) was examined for insertion mutants with defects in the biosynthesis of specific PAMPs. As expected, no transposon insertions in cell wall and lipoteichoic acid biosynthesis genes were identified, because these genes are required for staphylococcal growth (17). Lipoproteins are synthesized in the cytoplasm as precursors with an N-terminal signal peptide for secretion via the Sec pathway (18, 19). Lipoprotein diacylglycerol transferase (Lgt) catalyzes transfer of phosphatidylglycerol to the sulfhydryl moiety of a cysteine residue conserved in the signal peptides of all lipoprotein precursors (20, 21). The product of this reaction is then cleaved at the modified cysteine by lipoprotein (type II) signal peptidase (Lsp) (Fig. 1*B*) (22). In Gram-negative bacteria, the N-terminal cysteine residue of Braun's murein lipoprotein (23) is modified by *N*-acyltransferase (Lnt), yielding mature *N*-acylated lipoprotein (20). *Bursa aurealis* insertions in *lgt* and *lsp* were identified in the Phoenix library; however, bioinformatic analysis revealed that *lnt* is not present in the genome of *S. aureus*. Thus, the amine of cysteine-diacylglycerol lipoprotein is likely not acylated in staphylococci.

Genetic Requirement for Processing of Staphylococcal Lipoproteins. Wild-type *S. aureus* strain Newman grown in the presence of [^{3}H]palmitate incorporated radiolabeled palmitate into lipoproteins, however an isogenic *lgt* variant did not (see Fig. 6, which is published as supporting information on the PNAS web site). This defect is not caused by reduced protein synthesis, because labeling with [^{35}S]methionine revealed equal amounts of PrsA lipoprotein or its precursor in wild-type and *lgt* mutant staphylococci, respectively (see Fig. 7, which is published as supporting information on the PNAS web site). Furthermore, cells were labeled with [^{35}S]methionine for 1 min and PrsA was immunoprecipitated before and after a chase with nonradioactive methionine (see Fig. 8, which is published as supporting information on the PNAS web site). Wild-type bacteria synthesized a slower migrating precursor species (pro-PrsA) with an intact signal peptide that was converted to the mature form within 1 min.

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Abbreviations: LTA, lipoteichoic acid; PAMP, pathogen-associated molecule pattern; TLR, Toll-like receptor.

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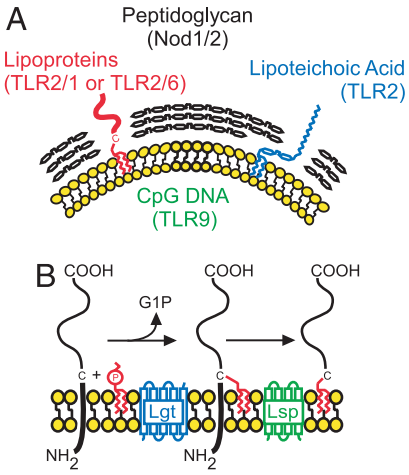

Fig. 1. Molecules of Gram-positive bacteria recognized by the innate immune system. (*A*) Schematic representation of proposed bacterial PAMPs to known TLRs and Nod1/2 (7, 8, 39). The plasma membrane and peptidoglycan are depicted in yellow and black, respectively. (*B*) Proposed pathway for lipoprotein maturation in *S. aureus*.

Signal peptide processing was blocked in *lgt* mutant staphylococci, showing accumulation of pro-PrsA. To determine whether *bursa aurealis* insertion in *lgt* affected both acylation (apo) and signal peptide cleavage, we compared *lgt* and *lsp* mutants. Pulse–chase labeling revealed that pro-PrsA processing did not occur in *lsp* mutants (Fig. 8, *lsp*), however this defect was restored upon expression of plasmid-encoded *lsp* (Fig. 8, *lsp*/pLsp). All biosynthetic defects were reversed upon transformation of *lgt* mutant staphylococci with the plasmid-encoded wild-type allele, indicating that the observed phenotypes are attributable to transposon insertion in the *lgt* gene (Fig. 8). Together, these data corroborate a model whereby Lgt-mediated acylation of prolipoprotein is a prerequisite for Lsp cleavage (20, 24). To examine the fate of apolipoproteins within cells, cultures were incubated for 5 min with [^{35}S]methionine, and bacteria were converted to protoplasts before immunoprecipitation with specific antibodies (see Fig. 9, which is published as supporting information on the PNAS web site). Processing of PrsA and GmpC lipoprotein precursors was blocked in *lgt* mutants, however slower migrating variants of PrsA and GmpC were found associated with protoplasts (P) or cell wall fractions (CW). Protoplast association corroborates the notion that hydrophobic signal sequences were not removed by Lsp without diacylglycerol acylation as displayed in Fig. 8. The localization of newly synthesized cell wall anchored protein A (Spa) and secreted staphylococcal nuclease (Nuc) was not affected by the disruption of *lgt*, indicating that the mutant strain did not cause a general defect in protein secretion.

Contribution of Lipoprotein Processing to Bacterial Recognition by Immune Surveillance Systems. We asked whether host recognition of *lgt* mutants by elements of the innate immune system was affected. Incomplete Freund's adjuvant-elicited mouse peritoneal macrophages were incubated with heat-killed staphylococci, and the production of proinflammatory cytokines was measured. *lgt* mutants induced significantly lower levels of TNF-α and IL-6 production than wild-type staphylococci (Fig. 2*A*). Complementation of this phenotype upon expression of wild-type *lgt* was reached within statistical significance (Fig. 2*A*, pLgt). The decrease in cytokine production was not due to macrophage cell death because no change in the number of viable cells was observed (data not shown). Similarly, the observed decrease in cytokine production was not secondary to alterations in the amount of lipoteichoic acid (LTA) or peptidoglycan, potent activators of the innate immune response (25–27). Wild-type and *lgt* mutant staphylococci appear to synthesize similar amounts of LTA, as determined by LTA extraction and immunoblotting (Fig. 2*C*). Release of IL-6 was measured in sera of infected animals for up to 6 h (Fig. 2*B*). Animals infected with *lgt* mutants failed to produce IL-6 compared with animals infected with wild-type staphylococci. These data are in agreement with a recent report showing that proinflammatory cytokine responses in various human cell lines are decreased upon incubation of *lgt* mutant compared with wild-type staphylococci (24). Because bacterial lipoproteins are known to engage TLR2, leading to cytokine production through NF-κB-dependent transcriptional activation, we used an NF-κB reporter assay system to assess the ability of wild-type and *lgt* mutant *S. aureus* to engage TLR2. Human 293 cell-stable transfectants, which express human TLR2, were transiently transfected with a reporter construct in which a NF-κB promoter drives expression of secreted alkaline phosphatase (SEAP).

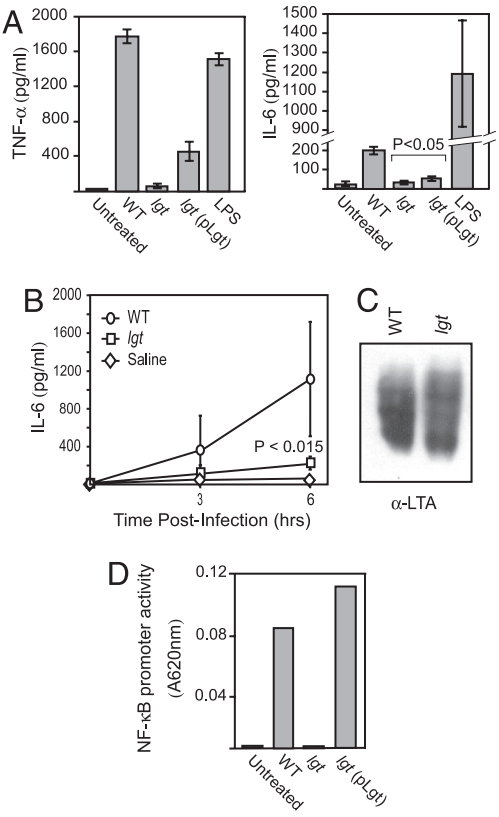

Fig. 2. Lack of recognition of *lgt* mutants by the innate immune system. (*A*) Incomplete Freund's adjuvant-elicited peritoneal macrophages were harvested from C57BL/6 mice and treated with either heat-killed staphylococci (5×10^6 cfu/ml) or LPS (0.1 μg/ml) for 18 h or untreated. The production of TNF-α (*Left*) and IL-6 (*Right*) was measured by ELISA. LPS was used as a control. (*B*) Serum cytokine production in response to infection with live staphylococci *in vivo*. Fifteen mice infected with 5×10^6 cfu of bacteria were anesthetized, and three mice were terminally bled at 0, 3, and 6 h after infection, respectively. Sera were collected by centrifugation after clotting and assayed by ELISA for IL-6. (*C*) *lgt* mutants produce normal amounts of LTA. Saturated staphylococci cultures were disrupted by using a bead beater, and insoluble material was resuspended in 4% SDS and separated by SDS/PAGE, followed by immunoblot analysis using anti-LTA antibody. (*D*) 293 cells expressing TLR2 were transfected with a secreted alkaline phosphatase (SEAP) reporter plasmid under the control of an NF-κB inducible promoter. Coculture of these cells with wild-type (WT) *S. aureus* revealed induction of NF-κB promoter activity, whereas coculture with *lgt* mutant *S. aureus* failed to induce NF-κB promoter activity. Plasmid-encoded lgt (*lgt*/pLgt) restored TLR2-mediated NF-κB promoter activity.

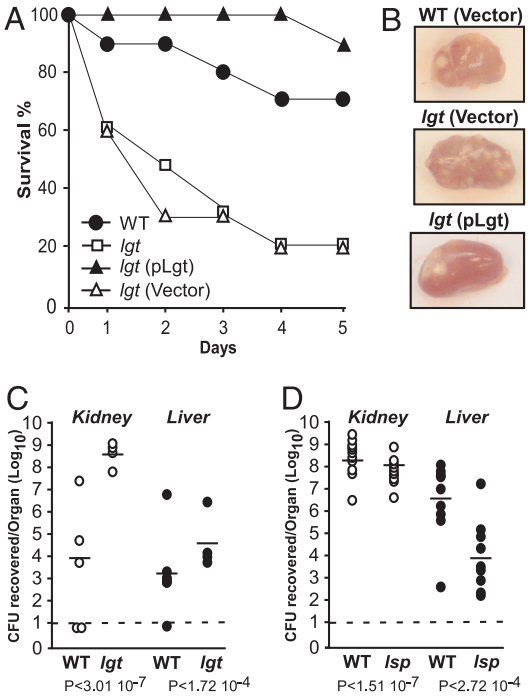

Fig. 3. Virulence of *S. aureus lgt* and *lsp* mutants. Six-week-old C57BL/6 mice were infected i.v. with 5×10^6 cfu for each strain. (*A*) Mice were monitored for the development of acute, lethal disease for 5 days upon infection of wild-type (WT) Newman, the *lgt* mutant strain, or *lgt* mutant carrying empty vector or complementing plasmid pLgt. (*B*) Kidneys were harvested from mice infected for 4 days with mutant strain *lgt* carrying the empty vector or infected for 5 days with WT Newman or complemented mutant strain *lgt*/pLgt. The kidneys were photographed to visualize the formation of abscesses. (*C*) In a separate experiment, animals infected with either WT or *lgt* mutant staphylococci and still alive 2 days after infection were killed. Kidneys and liver were removed and homogenized. Viable bacteria were counted after dilution and colony formation on tryptic soy agar. Statistical significance was examined with Student's *t* test, and *P* values were recorded. The limit of detection (dashed line) was determined to be 100 cfu (10^2). (*D*) Dissemination to organs of WT or *lsp* mutant staphylococci 4 days after infection.

Cells were cocultured with wild-type or *lgt* mutant bacteria in medium containing colorimetric substrate for SEAP detection. As demonstrated in Fig. 2*D*, NF-κB activity was detected in 293TLR2 cells cocultured with wild-type *S. aureus* Newman. However, no activity was detected in 293TLR2 cells that remained uninfected or when these cells were cocultured with *lgt* mutant bacteria. Restoration of *lgt* expression by way of plasmid complementation (*lgt*/plgt) revealed levels of NF-κB induction similar to that induced by coculture with wild-type *S. aureus*. Importantly, no NF-κB inducible activity was present in parental 293 cells that lack TLR2 (data not shown). Together, these data strongly suggest that *S. aureus*-derived bacterial lipoproteins function through TLR2 in an NF-κB dependent fashion to induce the production of inflammatory cytokines.

Virulence of *S. aureus lgt* and *lsp* Mutants. Wild-type *S. aureus* Newman or its isogenic *lgt* and *lsp* mutants (5×10^6 staphylococci) were administered i.v. via retro-orbital injection into mice. Disease progression was observed over 5 days, after which the animals were killed, and their internal organs were removed and inspected for abscess formation. Homogenized tissues were spread on agar medium and staphylococcal load within organ tissues enumerated by colony formation. In contrast to the sublethal infections of wild-type staphylococci that are eventually cleared by infected mice (Fig. 3*A*, filled circles), *bursa aurealis* insertion in *lgt* caused a rapid and pronounced mortality in infected animals over the experimental time course (Fig. 3*A*, open squares). This hypervirulent phenotype was not observed when *lgt* mutant strains carried the complementing pLgt plasmid (Fig. 3*A*, filled triangles). In contrast, mice infected with *lgt* mutant bacteria carrying only the vector control plasmid succumbed much more rapidly to infection (Fig. 3*A*, open triangles). Inspection of kidneys from animals infected with wild-type *S. aureus* revealed the presence of multiple raised, yellow lesions on the organ surface (Fig. 3*B*) that harbor collections of staphylococci and associated cellular debris. These lesions were more numerous in animals that had been infected with *lgt* mutant staphylococci. Animals infected with the complemented *lgt* mutant displayed gross pathology similar to that of the wild-type infected animals. Bacterial load in the organs of animals killed 48 h after infection was quantified. Data in Fig. 3*C* show that staphylococci lacking *lgt* proliferate to much higher numbers during infection compared with wild-type *S. aureus* (differences of 4.5 log in the kidneys and 1 log in the liver). Mice infected with *lsp* mutants did not develop acute, lethal disease (data not shown) and quantification of bacteria within infected organs (4 days after infection) demonstrated that this mutant displayed attenuated virulence with a severe defect in the ability to multiply in liver tissue (Fig. 3*D*). Previous studies demonstrated that lipoprotein processing by Lsp is required for the full virulence of *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* in animal models of infection (28, 29). Additionally, a signature-tagged mutagenesis screen of *S. aureus* identified *lsp* as a factor contributing to virulence; however, the calculated LD_{50} of this *lsp* mutant in an animal model of i.p. infection was found to be similar to that of the wild-type parental strain (30). Thus, the increased virulence of *lgt* mutants appears to be solely caused by the lack of Lgt activity that results in a loss of lipoprotein acylation, not signal sequence removal.

Growth of *S. aureus lgt* Mutant *in Vivo*. We wondered whether the proliferation of *lgt* mutant staphylococci could be the result of increased proliferation in the blood or increased resistance to phagocytosis. To distinguish between these possibilities, wild-type and *lgt* mutant bacteria were grown in the presence of fresh human whole blood, activated J774 murine macrophages, or freshly prepared human serum (see Fig. 10, which is published as supporting information on the PNAS web site). The results showed that *lgt* mutant bacteria proliferated much more slowly in the presence of blood or activated macrophages than *S. aureus* wild-type strain Newman but demonstrated similar proliferation in serum. These data suggest that the increased virulence observed in animals infected with the *lgt* mutant bacteria is not attributable to either enhanced proliferation or impaired clearance of the mutant bacteria. Furthermore, phagocytosis and macrophage killing of *lgt* mutants was not reduced compared with wild-type staphylococci, indicating that the mutant strain is not able to escape from phagocytic killing (data not shown). We sought to ascertain whether hypervirulence of *lgt* mutants may be caused by changes in exoprotein secretion or other traits associated with increased invasiveness. To examine this possibility, groups of 20 mice were inoculated i.p. either with buffer (PBS) or with 5×10^8 heat-killed bacteria of the wild-type or *lgt* mutant strains. This preinoculation with heat-killed wild-type or *lgt* mutant bacteria induced the generation of an IL-1 response in the host, detectable in peritoneal washes at 24 h (data not shown). Twenty-four hours after preinoculation, each group was divided into two groups of 10 mice, and cultures of *lgt* mutant or wild-type *S. aureus* Newman (5×10^6 staphylococci) were administered i.v. via retro-orbital injection. Disease progression was observed over 6 days (Fig. 4). Animals that were pretreated with the PBS alone developed acute, lethal disease upon i.v. challenge with the *lgt* mutant. However, pretreatment with heat-killed bacteria, either wild-type or mutant, resulted in

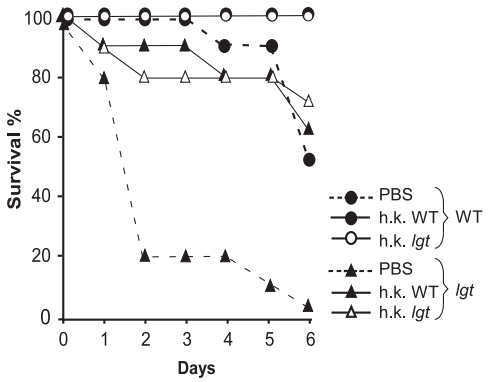

Fig. 4. Pretreatment with heat-killed staphylococci protects animals from infection. Mice were pretreated with i.p. injection of either buffer (PBS) or heat-killed wild-type (WT) or lgt mutant bacteria 24 h before i.v. challenge with live (5×10^6) WT or lgt mutant bacteria. The percentage of survival after challenge was recorded over a 6-day time course.

protection against hypervirulence of *lgt* mutant or even killing by wild-type staphylococci. These results suggest that *lgt* mutants *per se* have not acquired a factor or trait that precipitates a hypervirulent state. Furthermore, heat-killed bacteria, administered in large quantity into the peritoneal cavity, do stimulate innate host defenses capable of containing infections caused by *lgt* mutants.

Physiological Response to *S. aureus lgt* Mutant Infection. To probe the pathological consequence of infection, kidneys of animals infected with *S. aureus* wild-type Newman or *lgt* variants were removed 2 days after infection, formalin-fixed, and subsequently processed for microscopic evaluation of hematoxylin-eosin staining of thin sections (Fig. 5). As expected, infection with *S. aureus* strain Newman generated multiple small staphylococcal foci circumscribed by large numbers of infiltrating neutrophils. This zone of infection and inflammation is contained by a surrounding cuff of fibrin deposition. Surprisingly, physiologic host responses to infection with the *S. aureus lgt* mutant were abolished, exemplified by large collections of staphylococci in the kidney tissue with minimal neutrophil infiltration.

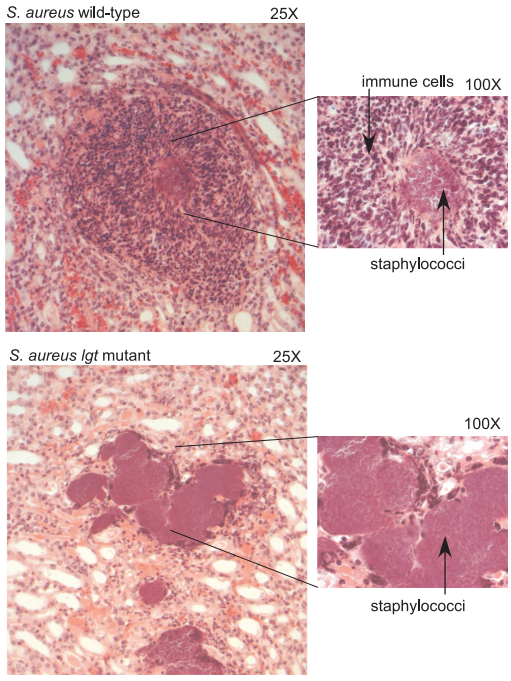

Fig. 5. Pathological substrate of infection caused by *S. aureus* wild-type and *lgt* mutant strains. Kidneys of 6-week-old C57BL/6 mice infected with 5×10^6 cfu *S. aureus* Newman (wild-type) or its isogenic *lgt* variant were analyzed 2 days after infection. Formalin-fixed tissues were embedded, sectioned, stained with hematoxylin/eosin, and viewed at ×25 (*Left*) and ×100 (*Right*) magnification.

Discussion

S. aureus is a physiological commensal of the human skin and nares (31). Breaches in local defense, such as a skin cut or hair follicle trauma, provide this pathogen with an opportunity to gain access to deeper tissues. *S. aureus* is capable of causing infections of any organ tissue. These infections may culminate in life-threatening bacteremia. Despite medical advances, the frequency of both community- and hospital-acquired *S. aureus* infections has increased steadily, and the treatment of these infections is becoming even more difficult with the emergence of antibiotic-resistant strains. This increased emergence of antibiotic resistance necessitates the identification of novel therapies that are capable of interfering with the virulence of multidrug-resistant strains of *S. aureus*.

The establishment of staphylococcal abscesses with liquefaction necrosis represents the sum of all pathogenetic events implemented by the activity of virulence factors, bacterial molecules sampled by the host and the corresponding host responses (32–34). The innate immune system plays an integral role in determining the outcome of the infection. Virulence studies using knockout mice have shown that both TLR2 and the signaling molecule MyD88 play a critical role in the innate immune response to staphylococcal infection (9), suggesting that TLR2-recognition of one or more staphylococcal PAMPs results in signaling through this adaptor molecule.

We have examined the contribution of staphylococcal lipoproteins during infection by targeting the only two genes surmised to be involved in this process, *lgt* and *lsp* (Fig. 1*B*). Taken together, our experiments reveal that *lgt* mutants that lack diacylglycerol-modified lipoproteins, but not *lsp* variants that accumulate uncleaved modified lipoproteins, escape detection by host innate immune surveillance systems. This result is surprising, because bacteria elaborate many different pattern molecules (peptidoglycan, teichoic acid, *N*-formyl methionine), each of which was hitherto thought sufficient to activate innate immune responses. In concert with our data, the recent finding that interleukin-1 receptor/MyD88 (but not TLR2) signaling pathways are essential for neutrophil recruitment and host responses to staphylococcal infection (35) highlights the complexity of the host/pathogen interaction. Staphylococcal diacylglycerol lipoproteins are therefore not only required for bacterial transport reactions (36) but are also essential in triggering the host innate response to infection. Because early innate responses to an invading pathogen provide a critical template for adaptive immune responses, staphylococcal lipoproteins assume a central role in defense against this pathogen. These results suggest further that immunomodulatory therapies with a diacylglycerol lipoprotein may be useful for treatment or prevention of human infections caused by *S. aureus*.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. *Escherichia coli* and *S. aureus* were grown in Luria–Bertani broth and tryptic soy broth, respectively, at 37°C. Chloramphenicol and erythromycin were used at 10 mg/liter, and ampicillin was used at 100 mg/liter. *lgt*, *lsp*, *nuc*, *spa*, and *gmpC* mutants were obtained from the Phoenix (ΦNΞ) library (16). Each Phoenix isolate is a derivative of the clinical isolate Newman (16, 37). All *bursa aurealis* insertions were transduced into wild-type *S. aureus* Newman by using bacteriophage ϕ85. Additional alleles were generated by

replacing the *lsp* and *lgt* coding region in strain Newman with the *ermC* cassette by allelic exchange as described in ref. 38. The pLgt complementation plasmid was generated by cloning the *hprK* promoter (275 bp upstream of the *hprK lgt yvoF yvcD* translational start site) upstream of the *lgt* coding region in *E. coli–S. aureus* shuttle vector pOS1. pLsp was generated by cloning *lsp* downstream of the *hprK* promoter in pOS1.

Macrophage Assays. Three days after i.p. injection with incomplete Freund's adjuvant, peritoneal cavities of 6- to 8-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were washed with cold, serum-free Hanks' balanced salt solution. Cells were plated in triplicate at a density of 2×10^6 cells per well by using 24-well dishes and serum-free RPMI medium 1640. After 2 h of incubation at 37°C in an atmosphere with 5% CO_2, plates were carefully washed three times with prewarmed, serum-free medium to remove nonadherent cells and fresh RPMI medium 1640 containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 50 μM 2-mercaptoethanol. Macrophage cultures were treated with 5×10^6 cfu/ml washed, heat-killed staphylococci or 0.1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) in RPMI medium 1640 containing 10% FBS. Macrophage culture supernatants were collected 18 h after the addition of proteins and analyzed by ELISA for concentration of IL-6 (BD Biosciences, San Jose, CA) and TNF-α (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

Immunoblot Analysis of LTA. Saturated staphylococci cultures grown in tryptic soy broth for 12 h were disrupted by using a bead beater, and insoluble material was recovered by centrifugation at $16{,}000 \times g$, boiled in 4% SDS for 30 min to disrupt membranes, separated by SDS/PAGE, and analyzed by immunoblot using LTA-specific monoclonal antibodies (HyCult BioTechnology, Uden, The Netherlands).

NF-κB Reporter Assay. A total of 293 parental cells (293null) and 293 cells expressing the TLR2 receptor (293TLR2C.6; Invivogen, San Diego, CA) were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, L-glutamine, blasticidin, and normocin according to the manufacturer's protocol. On day 0, cells were counted and plated at a density of 1×10^6 cells per well in six-well plates with 3 ml of medium lacking antimicrobial supplements. On day 1, cells were transiently transfected with 5 μg of pNiFty2-secreted alkaline phosphatase (SEAP) plasmid DNA (Invivogen) by using Lipofectamine 2000 (15 μl of lipofectamine mixed with DNA; Invitrogen). On day 2, medium from transfected cells was aspirated and replaced with 1 ml HEK-Blue detection medium (Invivogen). Overnight cultures of staphylococci were diluted 1:100 into fresh medium and grown to OD_{660} 0.5 ($\approx 2 \times 10^8$ cfu/ml). Staphylococci were sedimented by centrifugation, washed, and suspended in PBS, and 1×10^7 cfu in 20 μl suspension were added to each well of transfected 293 cells, followed by an 18-h incubation. Medium was removed from the wells. Cells, staphylococci, and debris were sedimented by centrifugation at $13{,}000 \times g$ for 1 min, and absorbance of supernatant was measured at OD_{620} as a measure of NF-κB promoter activity.

Virulence Studies. *S. aureus* strains were grown at 37°C overnight in tryptic soy broth, diluted 100-fold in fresh broth, and incubated at 37°C until OD_{660} of 0.5. Cells were washed, diluted, and suspended in PBS, and 100 μl of bacterial suspension was injected i.v. into 6-week-old female C57BL/6 mice. Viable staphylococci were enumerated by colony formation on tryptic soy agar to quantify the infection dose ($\approx 5 \times 10^6$ cfu). At the indicated time points, i.e., days after challenge, mice were killed by CO_2 asphyxiation. Spleen, kidneys, and liver were removed, and organs were homogenized in 1 ml of 1% Triton X-100 in PBS. Dilutions of the homogenates were plated on agar for enumeration of viable staphylococci. Statistical data analysis was performed with Student's *t* test by using the software Analyze-it (Analyze-it Software, Leeds, U.K.). For histology, kidneys of infected animals were placed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin. For complementation experiments, mice were administered chloramphenicol in their drinking water (0.5 mg/ml) 24 h before infection and until the end of the experiment. For protection experiments, heat-killed bacteria (5×10^8 cfu) were inoculated into groups of 20 6-week-old C57BL/6 mice i.p., and 24 h later the animals were challenged with live bacteria in suspension injected i.v. The time to death was recorded over 6 days.

Supporting Information. For details regarding protein labeling and pulse–chase analysis, fractionation of radiolabeled staphylococci, and bacterial proliferation in the presence of human blood, cultured macrophages, or human serum, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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1. Lowy FD (1998) *New Engl J Med* 339:520–532.
2. Thakker M, Park, J-S, Carey V, Lee JC (1998) *Infect Immun* 66: 5183–5189.
3. Thomas CA, Li Y, Kodama T, Suzuki H, Silverstein SC, El Khoury J (2000) *J Exp Med* 191:147–155.
4. Jonsson IM, Mazmanian SK, Schneewind O, Bremell T, Tarkowski A (2003) *Microb Infect* 5:775–780.
5. Jonsson P, Lindberg M, Haraldsson I, Wadstrom T (1985) *Infect Immun* 49:765–769.
6. Medzhitov R, Preston-Hurlburt P, Janeway CAJ (1997) *Nature* 388:394–397.
7. Medzhitov R (2001) *Nat Rev Immunol* 1:135–145.
8. Takeda K, Kaisho T, Akira S (2003) *Annu Rev Immunol* 21:335–376.
9. Takeuchi O, Hoshino K, Akira S (2000) *J Immunol* 165:5392–5396.
10. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S (1999) *Immunity* 11:443–451.
11. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ (1999) *J Biol Chem* 274:17406–17409.
12. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D (1999) *J Immunol* 163:1–5.
13. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, Zychlinsky A (1999) *Science* 285:736–739.
14. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, Maitland M, Norgard MV, Plevy SE, Smale ST, *et al.* (1999) *Science* 285:732–736.
15. Hirschfeld M, Kirschning CJ, Schwandner R, Wesche H, Weis JH, Wooten RM, Weis JJ (1999) *J Immunol* 163:2382–2386.
16. Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, Missiakas DM (2004) *Proc Natl Acad Sci USA* 101:12312–12317.
17. Neuhaus FC, Baddiley J (2003) *Microbiol Mol Biol Rev* 67:686–723.
18. Inouye S, Wang S, Sekizawa J, Halegoua S, Inouye M (1977) *Proc Natl Acad Sci USA* 74:1004–1008.
19. von Heijne G (1989) *Protein Eng* 2:531–534.
20. Tokunaga M, Tokunaga H, Wu HC (1982) *Proc Natl Acad Sci USA* 79:2255–2259.
21. Gan K, Gupta SD, Sankaran K, Schmid MB, Wu HC (1993) *J Biol Chem* 268:16544–16550.
22. Choi DS, Yamada H, Mizuno T, Mizushima S (1986) *J Biol Chem* 261:8953–8957.
23. Braun V, Bosch V (1972) *Eur J Biochem* 28:51–69.
24. Stoll H, Dengjel J, Nerz C, Gotz F (2005) *Infect Immun* 73:2411–2423.

25. Morath S, Stadelmaier A, Geyer A, Schmidt RR, Hartung T (2002) *J Exp Med* 195:1635–1640.
26. Deininger S, Stadelmaier A, von Aulock S, Morath S, Schmidt RR, Hartung T (2003) *J Immunol* 170:4134–4138.
27. Inohara N, Chamaillard M, McDonald C, Nunez G (2005) *Annu Rev Biochem* 74:355–383.
28. Petit CM, Brown JR, Ingraham K, Bryant AP, Holmes DJ (2001) *FEMS Microbiol Lett* 200:229–233.
29. Sander P, Rezwan M, Walker B, Rampini SK, Kroppenstedt RM, Ehlers S, Keller C, Keeble JR, Hagemeier M, Colston MJ, *et al.* (2004) *Mol Microbiol* 52:1543–1552.
30. Mei JM, Nourbakhsh F, Ford CW, Holden DW (1997) *Mol Microbiol* 26:399–407.
31. Archer GL, Climo MW (2001) *N Engl J Med* 344:55–56.
32. Dinges MM, Orwin PM, Schlievert PM (2000) *Clin Microbiol Rev* 13:16–34, table of contents.
33. Novick RP (2003) *Mol Microbiol* 48:1429–1449.
34. Archer GL (1998) *Clin Infect Dis* 26:1179–1181.
35. Miller LS, O'Connell RM, Gutierrez MA, Pietras EM, Shahangian A, Gross CE, Thirumala A, Cheung AL, Cheng G, Modlin RL (2006) *Immunity* 24:79–91.
36. Skaar EP, Humayun M, DeBord KL, Schneewind O (2004) *Science* 305:1626–1628.
37. Duthie ES, Lorenz LL (1952) *J Gen Microbiol* 6:95–107.
38. Bae T, Schneewind O (2006) *Plasmid* 55:58–63.
39. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori M-A, Werts C, Boneca IG (2004) *EMBO Rep* 5:1000–1006.