Auxiliary role for D-alanylated wall teichoic acid in Toll-like receptor 2-mediated survival of Staphylococcus aureus in macrophages

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Summary

We previously reported that *Staphylococcus aureus* avoids killing in macrophages by exploiting the action of Toll-like receptor (TLR) 2 that leads to the c-Jun N-terminal kinase (JNK)-mediated inhibition of superoxide production. To search for bacterial components responsible for this event, a series of *S. aureus* mutants, in which the synthesis of cell wall was interrupted, were screened for the level of JNK activation in macrophages. Besides a mutant lacking the putative TLR2 ligand, lipoproteins, two mutant strains were found to activate the phosphorylation of JNK to a lesser extent than the parental strain, and this defect was recovered by acquisition of the corresponding wild-type genes. Macrophages that had phagocytosed the mutant strains produced more superoxide than those engulfing the parental strain, and the mutant bacteria were more efficiently killed in macrophages than the parent. The genes mutated, *dltA* and *tagO*, encoded proteins involved in the synthesis of D-alanylated wall teichoic acid. Unlike a cell wall fraction rich in lipoproteins, D-alanine-bound wall teichoic acid purified from the parent strain by itself did not activate JNK phosphorylation in macrophages. These results suggested that D-alanylated wall teichoic acid of *S. aureus* modulates the cell wall milieu for lipoproteins so that they effectively serve as a ligand for TLR2.

Keywords: bacterial infection; macrophages; phagocytosis; teichoic acids; Toll-like receptor 2

Introduction

Invading microbial pathogens compete with host organisms in the regulation of innate immunity.[1–5] They try to circumvent host immune responses to achieve an effective infection and prolonged survival through, for example, inhibition of signaling pathways for the activation of nuclear factor (NF)-κB and mitogen-activated protein kinases, which induce the transcription of genes coding for antimicrobial substances and pro-inflammatory cytokines.[1–3] Some bacteria evade phagocytosis by immune cells or do not even concede once phagocytosed: they inhibit phagosomal maturation or escape from phagosomes to avoid digestion by lysosomal enzymes.[6] To overcome such microbial actions against immune responses, host immune cells adopt an alternative strategy, as exemplified by the induction of autophagy, in which cytoplasmic bacteria are resealed with membranes and subjected to lysis.[7–9] It is important to clarify the mechanism underlying the combat between microbial pathogens and host organisms for developing novel and effective medicines against infectious diseases.

We previously reported that *Staphylococcus aureus* inhibits the production of superoxide in macrophages to evade killing after phagocytosis, through Toll-like receptor (TLR) 2-mediated phosphorylation of c-Jun N-terminal kinase (JNK).[10] This is unique in that bacteria exploit the actions of innate immune receptors that recognize microbial pathogens, TLR2 in this case, for their survival in macrophages. Presumably, TLR2 is activated by a component(s) of *S. aureus* residing at the cell wall, such as lipoproteins and lipopeptides[11–17] with some controversies[18], to transmit a signal leading to the phosphorylation of JNK and subsequent inhibition of superoxide production in macrophages. In the present study, we took a genetic approach to search for additional bacterial components required for the exploitation of TLR2 by *S. aureus* and obtained evidence that genes responsible for the

synthesis of D-alanylated wall teichoic acid (WTA) play a crucial role.

Materials and methods

Materials

An antibody (#9251) specifically recognizing the phosphorylated form of JNK and another (#9252) recognizing both the phosphorylated and unphosphorylated forms were purchased from Cell Signaling Technology (Beverly, MA). Using these antibodies, two isoforms of JNK with M_r of 46 and 54 kDa, and their phosphorylated forms were detectable. pHY300PLK, an *Escherichia coli-S. aureus* shuttle vector containing a tetracycline-resistant gene, was obtained from Takara-Bio (Ohtsu, Japan). Fluorescein isothiocyanate was purchased from Molecular Probes (Eugene, OR); the synthetic lipopeptide tripalmitoyl-*S*-glycerylcysteine (Pam3Cys), lipopolysaccharide (LPS) from *Salmonella enteritidis,* and *N*-acetyl-L-cysteine were from Sigma-Aldrich (St. Louis, MO); mannitol salt agar medium was from Nissui (Tokyo, Japan); Diogenes was from National Diagnostics (Atlanta, GA); and the Dual Luciferase Assay kit was from Promega Corp. (Madison, WI).

Generation of bacterial strains, bacterial culture, and preparation and maintenance of animal cells

Cell surface mutants of *S. aureus* are derivatives of the parental wild-type *S. aureus* strain RN4220 (a derivative of NCTC8325-4, a restriction and *agr* mutant[19]) (Table 1). To construct the mutant strains M0614 and M0615, sequences corresponding to portions of the *SA0614* and *SA0615* genes (nucleotide positions 50–400 and 32–507, respectively, with the first nucleotide of the translation start codon numbered as 1) were amplified by PCR and inserted into the *S. aureus* integration vector pSF151.[20] RN4220 was then transformed with

the resulting plasmids pSF*SA0614* and pSF*SA0615*, and M0614 and M0615 where the cognate genes had been disrupted by homologous recombination were selected. RN4220 and all the mutant strains were grown with Luria-Bertani medium at 37˚C (except for M0702 that was grown at 30˚C) to full growth, washed once with PBS, and used in the subsequent experiments. Macrophages from the peritoneal cavity of thioglycollate-injected C57BL/6 mice were prepared and maintained with RPMI1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37˚C with 5% (v/v) CO_2 in air.[21] Mice carrying disrupted *tlr2* in a C57BL/6 background[22] were provided by Dr. Shizuo Akira of Osaka University. HEK293 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37˚C with 5% CO_2 in air. All the experiments involving animals were conducted according to protocols that had been approved by the Committee on Animal Experimentation of Kanazawa University.

Preparation of WTA and lipoproteins

WTA of *S. aureus* that retained D-alanine was prepared as described below. Bacteria were disrupted using glass beads and centrifuged at 800 × *g* for 10 min. The supernatants were re-centrifuged at 20,000 × *g* for 10 min, and the precipitates were suspended with 20 mM sodium citrate (pH4.7) containing 0.5% (w/v) SDS, heated at 60°C for 30 min, and centrifuged at 20,000 × *g* for 10 min. The precipitates were suspended with 5% (w/v) trichloroacetic acid, kept at room temperature for 18 h, and centrifuged at 20,000 × *g* for 10 min. The supernatants were mixed with acetone, and the resulting precipitates were dissolved with water and centrifuged as above. The final supernatants were collected as purified WTA. The purity of this WTA preparation was determined based on the amount of phosphorus contained in a given dry weight as well as by PAGE followed by staining with silver, according to standard procedures.[23,24] To examine the attachment of D-alanine, the WTA

preparation was incubated with 0.1 M NaOH at 37˚C for 2 h and separated in thin-layer chromatography on Silica-gel 60 with a solvent of *n*-propanol : pyrdine : acetic acid : water = 18 : 10 : 5 : 16, and the developed plate was treated with the ninhydrin reagent to visualize amino groups. A fraction rich in lipoproteins was prepared by the Triton X-114 phase-partitioning method, as described previously.[14] Briefly, cell lysates were treated with Triton X-114 (2% (v/v)) and centrifuged at 10,000 × *g* for 10 min at 37˚C, and materials in the Triton X-114 phase were precipitated with ethanol, dissolved with water, and used as the lipoprotein-rich fraction.

Assays for JNK phosphorylation, phagocytosis of S. aureus*, colony formation of engulfed* S. aureus*, superoxide production, and lysosomal enzyme*

The level of phosphorylated JNK was determined by Western blotting as described.[10] In brief, mouse peritoneal macrophages from either wild-type or *tlr2*-deficient mice were incubated with *S. aureus* (macrophages : bacteria = 1 : 5, except for wild-type macrophages with *tagO* and *lgt* mutants at a ratio of 1 : 10) or cell wall components at 37˚C and lysed in a buffer containing SDS and inhibitors of phosphatases and proteases, and the lysates were subjected to SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes and reacted with antibodies, and specific signals were visualized by a chemiluminescence reaction and processed using Fluor-S MultiImager (Bio-Rad, Hercules, CA). Phagocytosis reactions with peritoneal macrophages and fluorescein isothiocyanate-labeled *S. aureus* as the phagocytes and targets (macrophages : bacteria = 1 : 10), respectively, were carried out as described previously.[10] After incubation, the macrophage cultures were washed with PBS to remove unengulfed bacteria, and macrophages attached to culture containers were fixed and treated with trypan blue for quenching fluorescence from bacteria remaining attached to macrophages. The samples were then

examined by phase-contrast and fluorescence microscopy for the level of phagocytosis. To determine the values of colony-forming units of engulfed *S. aureus*, macrophages incubated with bacteria (macrophages : bacteria = 1 : 500) for 30 min were washed to remove unengulfed bacteria and further incubated for 30 min. The macrophages 0 and 30 min after the wash were lysed with water, and the lysates at serial dilutions were seeded on agar-solidified mannitol salt medium or Luria-Bertani medium, the latter of which contained tetracycline and was used for bacteria transformed with pHY300PLK-based plasmid. The plates were incubated overnight at 37˚C, and the number of colonies (only those surrounded by yellow rings with mannitol salt medium) was determined and presented relative to that obtained at time 0 after the wash. For the examination of superoxide production, macrophages maintained on coverslips with serum-free RPMI1640 medium were incubated with unlabeled bacteria (macrophages : bacteria = 1 : 1000) at 37˚C, and the amount of superoxide released into the culture medium was determined by a chemiluminescence reaction using Diogenes, as described previously.[10] For determining the activity of α-*N*-acetylglucosaminidase, whole-cell lysates of peritoneal macrophages were incubated with a reaction mixture containing 4-methylumbelliferyl *N*-acetyl-α-D-glucosaminide (Sigma-Aldrich), and the level of cleaved substrates was measured with a fluorometer, as described previously.[25]

Assay for NF-κB activation

HEK293 cells were transfected by the calcium/phosphate method overnight with a mixture of plasmid DNA including pELAM[26] (a gift from Dr. Douglas Golenbock at University of Massachusetts), a reporter gene vector expressing firefly luciferase under the control of a promoter activated by NF-κB; pRL-TK (Promega Corp.), a control reporter constitutively expressing Renilla luciferase used for the normalization of transfection efficiency; and mouse

TLR2 cDNA in pDisplay (Invitrogen, Carlsbad, CA) (a gift from Dr. Yoshiyuki Adachi at Tokyo University of Pharmacy and Life Science).[27] The cells were further cultured with fresh medium for a day and subsequently incubated with *S. aureus* for 2 h, and the cell lysates were examined for the amounts of firefly luciferase and Renilla luciferase using the Dual Luciferase Assay kit. The ratio of firefly luciferase to Renilla luciferase was determined and considered as the level of NF-κB activation.

Statistical analysis

Data are representative of at least 3 independent experiments (n = 2–3 in each experiment) that yielded similar results. Data from quantitative analyses are expressed as the mean ± standard deviations of the results from at least 3 independent experiments. Statistical analyses were performed using Student's *t*-test, and *P* values of less than 0.05 were considered significant. The data significantly different from controls were marked with asterisks.

Results

Inefficient JNK phosphorylation in macrophages after incubation with *S. aureus* lacking expression of *dltA* or *tagO*

To search for components of *S. aureus* responsible for the activation of TLR2-mediated phosphorylation of JNK in macrophages, we screened a series of *S. aureus* strains with mutations that affect the structure of cell wall (Table 1). Peritoneal macrophages from thioglycollate-injected mice were incubated with either the parental strain RN4220 or its mutant strains, and whole cell lysates were subjected to Western blotting to determine the level of the phosphorylated form of JNK. Macrophages showed a rise in the level of

phosphorylated JNK 10 min after the incubation with RN4220, and the increase continued during the next 20 min (left panel in Fig. 1a), as we reported previously.[10] Incubation with a mutant strain lacking the expression of *dltA* similarly brought about the activation of JNK phosphorylation, but the level was much lower than that observed with the parental strain (left panel in Fig. 1a). This effect was not due to impaired phagocytosis of the mutant bacteria by macrophages because the parental and mutant strains were comparable in their susceptibility to phagocytosis (right panels in Fig. 1a). The level of phosphorylated JNK was lower in macrophages incubated with the strain T013 (Fig. 1b), in which the *lgt* gene coding for lipoprotein diacylglycerol transferase is disrupted.[14] This mutant strain is devoid of lipid modification of all lipoproteins at the cell surface, and the result was in line with previous reports that lipoproteins serve as a ligand for TLR2. Similar reduction in the level of JNK phosphorylation was seen when macrophages were incubated with a *tagO*-deficient strain and, though less significantly, mutants for the genes *SA0614* or *SA0615* (Fig. 1b). The other mutant strains including one deficient in the *ltaS* gene, which codes for polyglycerolphosphate synthase of lipoteichoic acid (LTA), did not differ from the parental strain as to the effect on the phosphorylation of JNK in macrophages (Fig. 1b). When macrophages were incubated with the *dltA* mutant that had been introduced with a plasmid expressing the *dltABCD* operon, the level of phosphorylated JNK became almost equal to that in macrophages incubated with the parental strain (left panel in Fig. 1c). Similarly, the expression of *tagO* in the *tagO* mutant complemented a defect in the phosphorylation of JNK (right panel in Fig. 1c). These results confirmed the importance of *dltA* and *tagO* for *S. aureus* to induce JNK phosphorylation in macrophages.

Auxiliary action of D-alanylated teichoic acid in the activation of TLR2 by *S. aureus*

Unlike TLR4-acting LPS, the parent and mutant strains deficient in *dltA* or *tagO* did not seem to activate macrophages lacking the expression of TLR2 in terms of the induction of JNK

phosphorylation (Fig. 2a). This indicated that the *S. aureus*-activated phosphorylation of JNK depends on the action of TLR2. In fact, a fraction rich in lipoproteins, a putative ligand for TLR2, obtained from the parent strain, the *dltA* mutant, and the *tagO* mutant, but not from a mutant strain lacking the expression of *lgt*, induced the phosphorylation of JNK in wild-type macrophages (Fig. 2b).

The *dltA* gene codes for one of the proteins responsible for the D-alanylation of teichoic acids,[28] and *tagO* codes for an enzyme responsible for the transfer of *N*-acetylglucosamine phosphate to the lipid carrier,[28] an essential step in the synthesis of WTA. On the other hand, *SA0614* and *SA0615* code for proteins that compose a two-component system of *S. aureus*, which induces the expression of the *dltABCD* operon.[29,30] Considering the results with mutant strains deficient in these genes as well as one with the *ltaS* mutant lacking LTA, we anticipated that D-alanylated WTA is required for the TLR2-mediated phosphorylation of JNK in macrophages. To more directly determine the role of WTA, we isolated WTA from *S. aureus* strains and examined its action. The WTA preparation was considered to be nearly pure based on the content of phosphorus and the staining pattern in PAGE (left and middle panels in Fig. 2c): note that none was obtained from the *tagO* mutant lacking an enzyme essential for WTA synthesis, and that a difference in the migration of WTA prepared from the *dltA* mutant was probably due to a lack of D-alanine. In fact, WTA of the *dltA* mutant strain seemed to be devoid of D-alanine whereas that of the parental and the *lgt* mutant strains retained it (right panel in Fig. 2c). When macrophages were incubated with these WTA preparations, the phosphorylation of JNK was not induced irrespective of the presence of bound D-alanine in WTA (Fig. 2d), indicating that WTA does not serve as a ligand for TLR2. We next tested if WTA influences the action of the TLR2 ligand. To this end, macrophages were incubated with Pam3Cys, a synthetic TLR2 ligand, in the presence and absence of WTA. However, the level of phosphorylated JNK was not altered by the addition of WTA (Fig. 2d). These results suggested that D-alanylated WTA

serves as an auxiliary factor for the TLR2 ligand in the context of bacterial cell wall so that it effectively acts on TLR2 to induce the phosphorylation of JNK.

Enhanced superoxide production and accelerated killing of engulfed bacteria in macrophages incubated with *S. aureus* deficient in *dltA* or *tagO*

We next determined the level of superoxide production in *S. aureus*-incubated macrophages, which we previously showed to be inhibited by phosphorylated and thus activated JNK.[10] The level of superoxide released from macrophages into culture media was significantly higher on incubation with a mutant strain lacking the expression of *dltA*, *tagO* or *lgt* than with the parental strain (left panel in Fig. 3a). The effect of the mutation in *dltA* was mitigated when the *dltABCD* operon was introduced into the mutant strain (right panel in Fig. 3a), confirming the requirement of *dltA* for the effective inhibition of superoxide production in macrophages by *S. aureus*. The viability of engulfed *S. aureus* was then assessed based on their colony-forming ability. The value of colony-forming units obtained with the *dltA* mutant was much smaller than that with the parental or the same mutant strain that had acquired the corresponding wild-type operon (Fig. 3b), indicating that *S. aureus* lacking the expression of *dltA* was more efficiently killed in macrophages. Furthermore, the *dltA* mutant survived the killing in macrophages when the cultures were supplemented with *N*-acetyl-L-cysteine, a superoxide scavenger (Fig. 3c). We next examined if the recognition and engulfment of *S. aureus* alters the activity of macrophages other than superoxide production. For this purpose, macrophages were incubated with various *S. aureus* stains, and their whole-cell lysates were assayed for α-*N*-acetylglucosaminidase, a major lysosomal enzyme. However, its activity did not change after the incubation with any of the bacterial strains tested (Fig. 3d), suggesting that the lysosomal activity is not influenced by *S. aureus*. These results indicated that a lack of the expression of *dltA* or *tagO* in *S. aureus* causes augmented production of superoxide and accelerated killing of engulfed bacteria in

macrophages, and thus suggested a role for the D-alanylation of WTA in the survival of *S. aureus* in macrophages.

Inefficient activation of NF-κB in TLR2-expressing cells incubated with *S. aureus* lacking *dltA* or *tagO*

We next determined the level of NF-κB-dependent gene expression in TLR2-expressing HEK293 cells, examining a role for *dltA* and *tagO* in the activation of TLR2. The expression of a NF-κB-induced gene coding for luciferase depended on the presence of TLR2 in HEK293 cells as well as the addition of *S. aureus* to them (Fig. 4a), indicating that the level of active NF-κB reflects the activation of TLR2-initiated signaling by bacteria. HEK293 cells incubated with the *dltA* mutant produced much less luciferase than those treated with the parental strain, and this decrease was recovered when the *dltABCD* operon was introduced into the mutant (Fig. 4b). A decrease in the level of active NF-κB was similarly observed when the mutants for *tagO*, *SA0614*, and *SA0615*, which all gave a reduced level of phosphorylated JNK in macrophages (see Fig. 1b), were tested (Fig. 4c). In contrast, the other mutant strains with no effect on the phosphorylation of JNK activated NF-κB as effectively as the parental strain (Fig. 4c). These results suggested that D-alanylated WTA is required for *S. aureus* to effectively induce the TLR2-mediated activation of NF-κB.

Taken together, the effects of *dltA* and *tagO* on JNK phosphorylation, superoxide production, the survival of engulfed bacteria, and the activation of TLR2-mediated signaling are consistent with the concept that a component of *S. aureus*, i.e. D-alanylated WTA, additional to an authentic ligand is required for TLR2 to effectively phosphorylate and activate JNK leading to the inhibition of superoxide production in *S. aureus*-engulfing macrophages.

Discussion

The study presented here showed that genes responsible for the synthesis and D-alanylation of teichoic acids are required for the TLR2/JNK-dependent survival of *S. aureus* in macrophages. The importance of D-alanylated LTA of *S. aureus* for the production of a pro-inflammatory cytokine by macrophages has been reported.[31] However, our results clearly indicated that WTA, not LTA, is necessary for the TLR2-mediated phosphorylation of JNK. There are preceding reports showing an *in vivo* role for the D-alanylation of teichoic acids of *S. aureus*[32] and *Streptococcus gordonii*[33] in bacterial virulence and TLR2-mediated host defense. Our study provides a reasonable explanation for the observation in these papers that bacteria evoking a higher level of immune response in host organisms are, at the same time, more infectious and virulent. We also showed that the D-alanylation of teichoic acids is necessary for *S. aureus* to effectively activate NF-κB in TLR2-expressing cells. It can thus be anticipated that D-alanylated WTA plays an important role in the TLR2-initiated signaling pathways in immune cells in favor of both host organisms and invading microbial pathogens. Purified WTA by itself did not induce JNK phosphorylation in macrophages, and exogenously added WTA was not effective on the phosphorylation of JNK induced by a synthetic ligand for TLR2. Therefore, it is probable that D-alanylated WTA does not directly act on TLR2 as a ligand but facilitates the activation of TLR2 by an authentic ligand like lipoproteins or lipopeptides in the context of the bacterial cell wall. There is a report showing that WTA mediates the interaction of *S. aureus* with airway epithelial cells.[34] However, this is not the case in our study because the level of the phagocytosis of *S. aureus* by macrophages does not differ between the parental and *dltA* mutant strains. We speculate that WTA modulates the cell wall milieu for lipoproteins so that they effectively serve as a ligand for TLR2.

The stimulation of JNK phosphorylation occurred when TLR2-lacking macrophages were incubated with LPS. This suggests that the JNK-mediated inhibition of killing of engulfed bacteria is not restricted to TLR2-stimulating bacteria, *S. aureus* in our study, but is observed for the bacteria recognized by TLR4. We previously reported that TLR4 delays the fusion between lysosomes and phagosomes that contain engulfed apoptotic cells.[25] Other investigators have also reported the involvement of TLR in the regulation of phagosome maturation and thus the fate of engulfed materials including microbial pathogens, microbe-infected cells, and apoptotic cells.[35–37] They argue that TLR-mediated control of phagosome maturation relates to the regulation of antigen presentation.[38–41] It is thus obvious that TLRs, TLR2 and TLR4 in particular, participate in not only humoral but also cellular immune responses. Further investigation will be necessary to obtain a complete picture that illustrates the mechanisms and consequences of TLR-mediated regulation of cellular immunity including phagocytosis.

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Figure legends

Figure 1. Inefficient phosphorylation of JNK in macrophages incubated with *S. aureus* lacking the expression of D-alanylated WTA. Mouse peritoneal macrophages were incubated with various *S. aureus* strains: the parental strain RN4220 (parent) and mutant strains lacking the expression of the indicated genes with the symbol 'Δ', and responses in macrophages were examined. (a) Macrophages were incubated with the parental strain or the mutant strain deficient in *dltA* for the indicated periods (30 min for phagocytosis), and the levels of phosphorylated JNK and phagocytosis of bacteria were determined. Arrowheads with the terms 'P-JNK' and 'total' denote the positions of the phosphorylated and both the phosphorylated and unphosphorylated forms of JNK, respectively. (b, c) Macrophages were incubated with the indicated *S. aureus* strains for 30 min, and the level of phosphorylated JNK was determined. In (c), the *S. aureus* strains used were the parental strain transformed with pHY300PLK alone (vector), Δ*dltA* transformed with pHY300PLK alone, Δ*dltA* transformed with *dltABCD* operon-expressing pHY300PLK (*dltABCD*), Δ*tagO* transformed with pHY300PLK alone, and Δ*tagO* transformed with *tagO*-expressing pHY300PLK (*tagO*).

Figure 2. Absence of JNK phosphorylation in macrophages treated with D-alanylated WTA. (a) Peritoneal macrophages of *tlr2*-disrupted mice were incubated with the indicated *S. aureus* strains or LPS (0.1 mg/ml) for 30 min, and the level of phosphorylated JNK was determined. (b) Macrophages of wild-type mice were incubated with a fraction rich in lipoproteins (0.1 μg/ml) obtained from the indicated *S. aureus* strains for 30 min, and the level of phosphorylated JNK was determined. (c) WTA prepared from the indicated *S. aureus* strains was characterized: determination of the phosphorus content (left), PAGE of WTA visualized with silver (middle), and thin-layer chromatography of D-alanine alkali liberated

from WTA (visualized by the ninhydrin reaction) (right). The arrowhead with 'D-Ala' in the right panel points to the position of D-alanine analyzed as a control. (d) Wild-type macrophages were incubated for 15 min with and without Pam3Cys (1 μg/ml), a synthetic TLR2 ligand, and WTA (10 μg/ml) purified from the indicated *S. aureus* strains, and the level of JNK phosphorylation was determined.

Figure 3. Increased production of superoxide and accelerated killing of engulfed bacteria in macrophages incubated with *dltA*-, *tagO*-, or *lgt*-deficient *S. aureus*. Mouse peritoneal macrophages were incubated with *S. aureus*, and the levels of superoxide production and values of colony-forming units of engulfed bacteria were determined. (a) Macrophages were incubated with the indicated *S. aureus* strains for 30 min, and the amount of superoxide in the culture medium was determined and presented relative (in percentage terms) to that in incubation with the parental strain RN4220 (left panel) or the *dltA* mutant transfected with the empty vector (right panel). (b) The value of colony-forming units of the indicated *S. aureus* strains that had been engulfed by macrophages was determined 0 and 30 min after elimination of unengulfed bacteria and shown relative (in percentage terms) to that obtained at time 0. (c) Macrophages that had been pre-treated with *N*-acetyl-L-cysteine (LNAC) (2 mM) or left untreated at 37˚C for 1 h were incubated with the indicated strains, and the value of colony-forming units of engulfed bacteria was determined. (d) Macrophages were incubated with the indicated *S. aureus* strains or left untreated (none), and their whole-cell lysates were assayed for the activity of α-*N*-acetylglucosaminidase at the indicated time points.

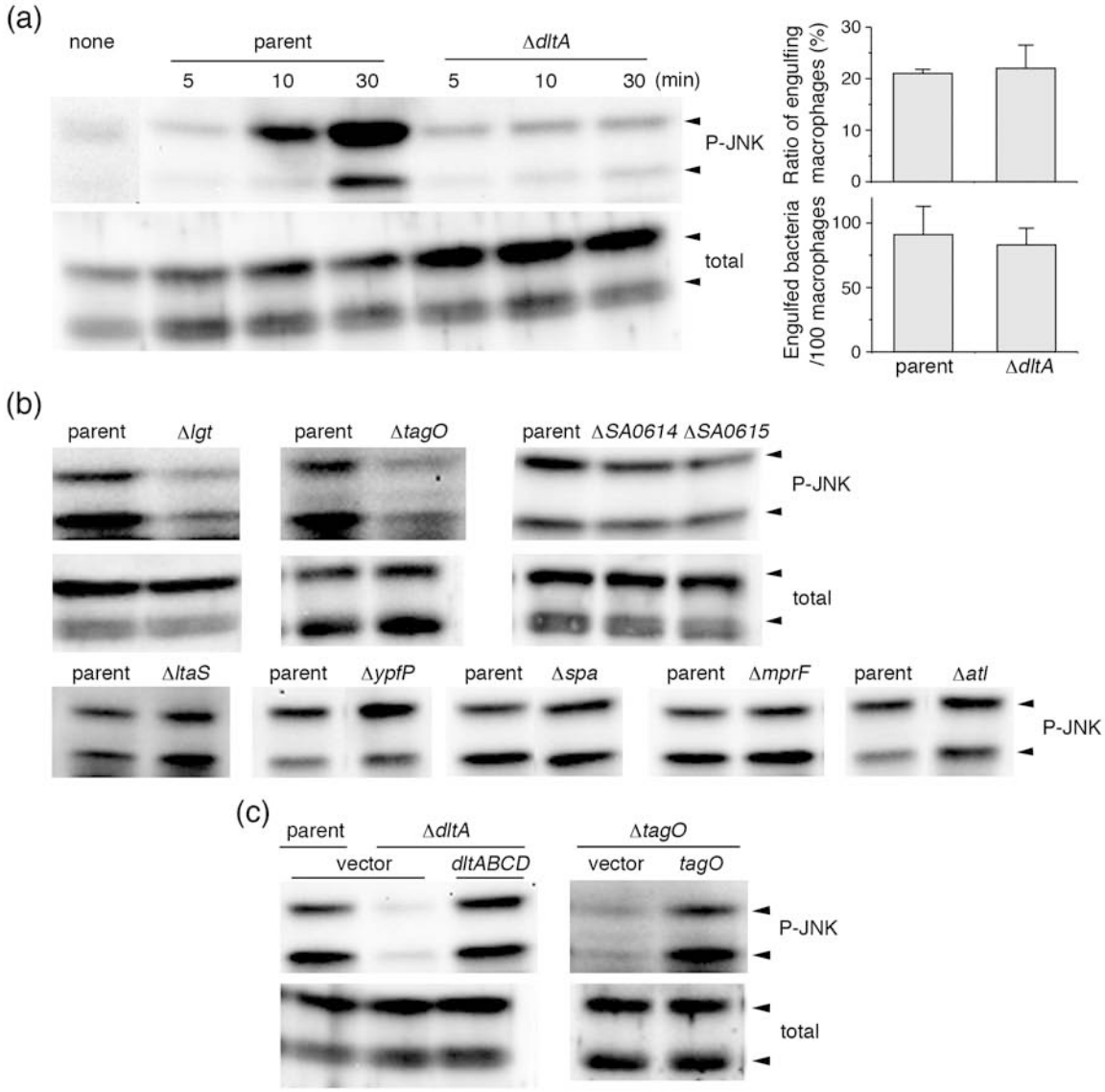
Figure 4. Inefficient activation of NF-κB in TLR2-expressing cells incubated with *dltA*-deficient *S. aureus*. (a) (left) HEK293 cells with and without TLR2 expression were incubated with RN4220 (HEK293 : bacteria = 1 : 10^3) or left untreated, and the level of

NF-κB activation was determined and shown relative to that in cells expressing TLR2 with no added bacteria, taken as 1. (right) HEK293 cells (1×10^5) expressing TLR2 were incubated with increasing amounts of RN4220, and the level of NF-κB activation was determined and shown relative to that in the experiment with no added bacteria, taken as 1. (b, c) HEK293 cells expressing TLR2 were incubated with the indicated *S. aureus* strains (HEK293 : bacteria = 1 : 100), and the level of NF-κB activation was determined and shown relative (in percentage terms) to that in the experiment with the parental strain RN4220.

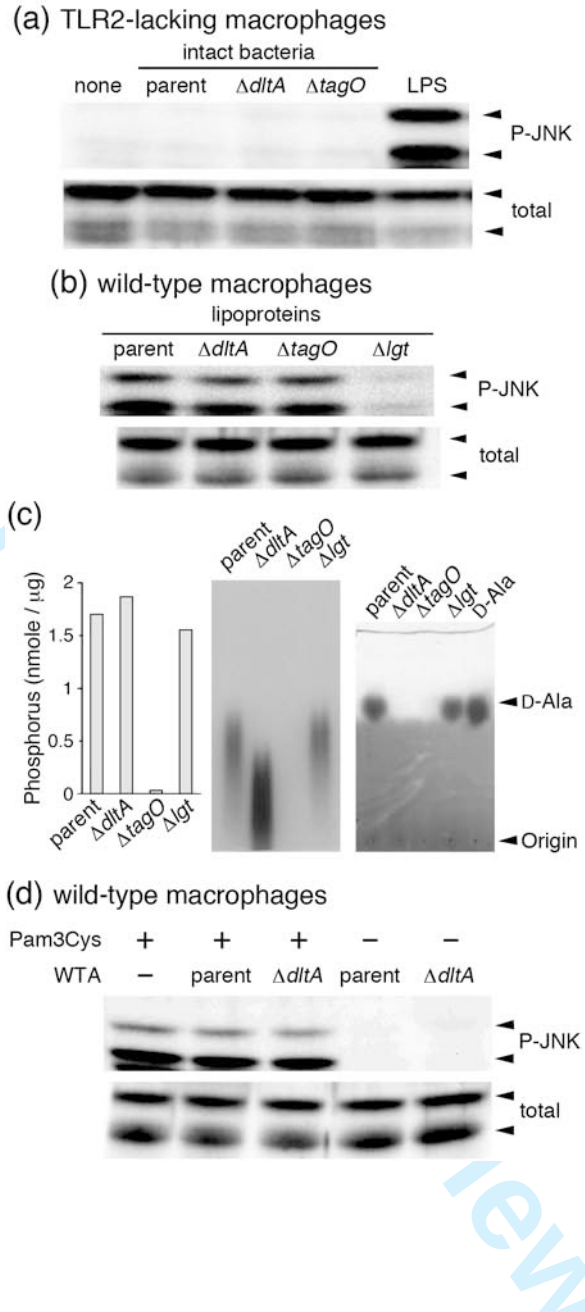
Table 1. *S. aureus* strains and plasmids used in this study.

Names	Genotypes and characteristics	References
RN4220	NCTC8325-4, restriction mutant	19
M0793	RN4220 *dltA*::pT0793	42
M0702	RN4220 *tagO*::pT0702	42
M0614	RN4220 *SA0614*::pSF*SA0614*	this study
M0615	RN4220 *SA0615*::pSF*SA0615*	this study
M0674/pM101	RN4220 *ltaS*::*phleo*/pM101	43
M0875	RN4220 *ypfP*::pT0875	42
JT1304	RN4220 *atl*::*cat*	44
CK1001	RN4220 *mprF*::pCK20*mprF*	45
M0107	RN4220 *spa*::*phleo*	unpublished observation
T013	RN4220 *lgt*::pM*lgt*	14
pHY300PLK	*E. coli-S. aureus* shuttle vector; *Amp*^{r} *Tet*^{r}	purchased from Takara-Bio
p0793	pHY300 with intact *dltABCD* from RN4220	42
p0702	pHY300 with intact *tagO* from RN4220	42
pSF151	*S. aureus* integration vector; *Km*^{r}	20
pSF*SA0614*	pSF151 with partial *SA0614* from RN4220	this study
pSF*SA0615*	pSF151 with partial *SA0615* from RN4220	this study

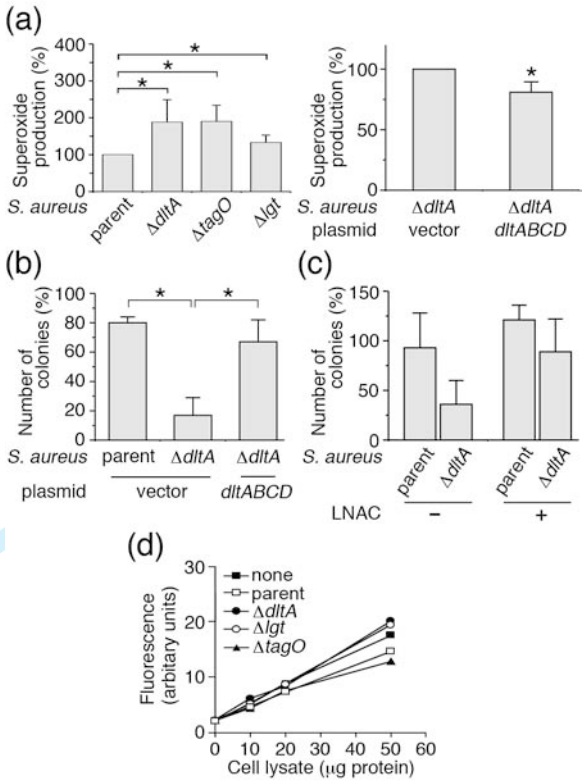
Shiratsuchi et al. Fig. 1



Shiratsuchi et al. Fig. 2



Shiratsuchi et al. Fig. 3



Shiratsuchi et al. Fig. 4