The innate immune system senses invading pathogens through a mechanism involving germ line-encoded pattern recognition receptors (1). Recent studies have revealed that the Toll-like receptors (TLRs)⁷ play an important role in recognizing microorganisms in mammals (2). To date, 11 human TLRs and 13 mouse TLRs have been identified, and each TLR appears to recognize a pathogen-associated molecular pattern derived from various microorganisms, including bacteria, viruses, protozoa, and fungi (2). Among these, TLR4 and TLR9 are essential for the responses to lipopolysaccharide and bacterial DNA, respectively (3, 4). The TLR2 ligand molecules that have been reported thus far are lipopolysaccharides from different bacterial strains, lipoproteins, synthetic lipopeptides, peptidoglycan (PGN), lipoteichoic acids (LTA), lipomannans, and lipoarabinomannans (2). Since these molecules are so structurally diverse, it appears unlikely that TLR2 has the capability to react with all agonists to the same degree. Several groups have recently provided clear evidence that the lipoproteins of bacteria function as a ligand for TLR2 based on studies using genetically engineered lipoprotein maturation-deficient bacteria from *Staphylococcus aureus*, *Listeria monocytogenes*, and Group B *Streptococcus* (5–8). These reports suggested the possibility that all other reported bacterial TLR2 ligand molecules were most likely due to contamination of highly active natural lipoproteins. However, these reports did not offer clues as to the biochemical nature of the native lipoproteins that act as TLR2 agonists.

Bacterial lipoproteins are structurally divided into two groups, diacylated lipoproteins and triacylated lipoproteins. These two groups have been distinguished by the absence or presence of a third enzyme involved in the maturation of bacterial lipoproteins (9–11). During the maturation process, the first enzyme, Lgt (lipoprotein diacylglycerol transferase), catalyzes the transfer of diacylglycerol to the sulfhydryl moiety of a cysteine residue conserved in the signal peptide of lipoprotein
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FIGURE 1. Identification of S. aureus 33-kDa TLR2-stimulating protein. A–H, the CHO/hCD14/hTLR2 reporter cell line harboring the NF-kB-driven hCD25 gene was stimulated for 16 h, and the resulting surface expression of CD25 was analyzed by flow cytometry. Bacteria were precultured at 30 °C in A and B or at 37 °C in C–E. The stimulation was induced by S. aureus whole cells of the parent RN4220 (A and C), by its LTA-deficient ΔltaS mutant (B), by its lipoprotein maturation-deficient Δlgt mutant harboring an empty plasmid (D), and by the Δlgt mutant harboring the pSltg plasmid (E). In A–E, the ratio of CHO cells to bacterial cells was 1:10 (blue) and 1:100 (magenta) in (A) and (B) and 1:5 (blue) and 1:50 (magenta) in C–E. Also, the CHO/hCD14/hTLR2 cells were stimulated with the soluble PGN fraction (F), with the Triton X-114 detergent fraction (G), and with its aqueous fraction after Triton X-114 treatment (H). The amount of soluble PGN used for cell stimulation was 0.7 μg/ml (blue) or 7 μg/ml (magenta) in F. The Triton X-114 fractions containing 24 ng (blue) or 240 ng (magenta) proteins were treated to cells (G). One-fold (blue) or 10-fold (magenta) aqueous fractions of soluble PGN amounts were used to treat cells (H). The black line with gray area indicates mock treatment (A–H). I, SDS-PAGE analyses of the soluble PGN fraction (60 μg dry weight; lane 1), aqueous phase (lane 2), and Triton X-114 detergent phase concentrated by 10-fold (lane 3), which were obtained from S. aureus RN4220 harboring the empty plasmid pKE515. The arrowheads and arrow indicate Protein A and 33-kDa protein, respectively.

precursors, whereas the second enzyme, Lsp (lipoprotein maturation-deficient Δlgt mutant harboring an empty plasmid (D), and by the Δlgt mutant harboring the pSltg plasmid (E). In A–E, the ratio of CHO cells to bacterial cells was 1:10 (blue) and 1:100 (magenta) in (A) and (B) and 1:5 (blue) and 1:50 (magenta) in C–E. Also, the CHO/hCD14/hTLR2 cells were stimulated with the soluble PGN fraction (F), with the Triton X-114 detergent fraction (G), and with its aqueous fraction after Triton X-114 treatment (H). The amount of soluble PGN used for cell stimulation was 0.7 μg/ml (blue) or 7 μg/ml (magenta) in F. The Triton X-114 fractions containing 24 ng (blue) or 240 ng (magenta) proteins were treated to cells (G). One-fold (blue) or 10-fold (magenta) aqueous fractions of soluble PGN amounts were used to treat cells (H). The black line with gray area indicates mock treatment (A–H). I, SDS-PAGE analyses of the soluble PGN fraction (60 μg dry weight; lane 1), aqueous phase (lane 2), and Triton X-114 detergent phase concentrated by 10-fold (lane 3), which were obtained from S. aureus RN4220 harboring the empty plasmid pKE515. The arrowheads and arrow indicate Protein A and 33-kDa protein, respectively.

ligand. Also, we show that an S. aureus SitC lipoprotein is triacylated, and SitC-mediated recognition signal is transferred via TLR1/TLR2 or TLR2/TLR6 heterodimer in mouse peritoneal macrophages.

EXPERIMENTAL PROCEDURES

Standard methods for maintenance of bacteria, manipulation of DNA, purification and analysis of lipoproteins, protein electroelution, and analyses of lipoprotein were used throughout. Full experimental procedures and any associated references are available in the supplemental material.

RESULTS

The Lipoprotein-deficient (Δlgt) S. aureus Mutant Cannot Activate TLR2, whereas the LTA-deficient (ΔltaS) Mutant Can Activate TLR2 in Vivo—In order to address which molecule(s) of the cell wall function(s) as a native TLR2 ligand, we studied two S. aureus mutant strains. One of them was an LTA-depleted ΔltaS mutant strain (17, 18), whereas the other was a Δlgt mutant that lacks Lgt, an enzyme that is responsible for catalyzing the diacylglycerol modification of lipoprotein precursors, leading to a block of the complete lipoprotein maturation process (8). When the ΔltaS mutant cells were incubated with TLR2-expressing CHO cells, the TLR2 stimulation ability of the ΔltaS mutant was very similar to that observed for the parent S. aureus RN4220 strain (Fig. 1, A and B). In contrast, unlike its parent strain (Fig. 1C), the Δlgt mutant was completely unable to induce TLR2 activation (Fig. 1D), which was recovered by an introduction of an lgt gene-harboring plasmid into the Δlgt mutant strain (Fig. 1E).

Analogous results were obtained if TLR2-mediated NF-κB promoter activity was observed on TLR2-expressing HEK293 cells using the ΔltaS and Δlgt mutant (Fig. S1, A and B). These results demonstrated that the maturation of lipoprotein(s) in S. aureus is essential for TLR2 stimulation and that LTA in S. aureus is not the primary molecule that stimulates TLR2, at least under our reaction conditions.

The Insoluble S. aureus PGN Contains 33-kDa TLR2-stimulating Protein—Recent bioinformatic analyses of the six S. aureus genomic sequences have assigned 47–55 genes as lipoprotein candidates in each complete genome (19). In addition, some of lipoproteins are predicted to localize in the PGN layer, since lipoproteins are anchored to the outer leaflet of the cell membrane via their lipid moieties (20). In order to determine which lipoprotein(s) in the S. aureus cell wall function(s) as a TLR2 ligand, we prepared the insoluble PGN fraction from S. aureus RN4220 cells. Although PGN from S. aureus has long been suggested as a potential TLR2 ligand, the loss of TLR2
stimulation activity in the S. aureus Δtgt mutant as shown in Fig. 1D suggests that unidentified lipoprotein(s) in the insoluble PGN fraction, but not PGN itself, activates TLR2. We hypothesized that we could purify the PGN-anchored lipoprotein(s) to homogeneity as a native TLR2 ligand if the insoluble PGN was first degraded enzymatically. We subsequently solubilized the PGN of S. aureus with Achromobacter. Surprisingly, the result showed a series of eight mass peaks harboring 14 mass differences (positions 1–8 in Fig. 2B) that corresponded to theoretical m/z of N-acylated-S-(diacyl-propyl)-cysteinyl-peptide and those of fatty acids. Consistently, we could obtain a homogenous 200-mg sample of the purified 33-kDa protein from an original 100-mg dry weight sample of insoluble PGN by using Triton X-114 phase partitioning and an electroelution from an SDS-polyacrylamide gel (see supplemental material).

The 33-kDa Protein Is Identified as Triacylated SitC Lipoprotein—In order to address the identity of the purified 33-kDa protein, we determined the internal amino acid sequences using a gas phase automatic amino acid sequencer in addition to implementing liquid chromatography-tandem mass spectrometry (MS/MS). The results indicated that the 33-kDa protein was encoded by the sitc gene (accession number YP_499195; Fig. 2A). The amino acid sequences of the three internal peptide fragments obtained by lysylendopeptidase digestion and stained with Coomassie Brilliant Blue, we obtained primarily a single 33-kDa protein band (Fig. 11, lane 3). In order to characterize this protein, we obtained a homogeneous 200-μg sample of the 33-kDa protein from an original 100-mg dry weight sample of insoluble PGN by using Triton X-114 phase partitioning and an electroelution from an SDS-polyacrylamide gel (see supplemental material).

**FIGURE 2.** The 33-kDa protein is identified as triacylated SitC lipoprotein. A, indicates the whole amino acid sequence of the S. aureus SitC protein (box, signal peptide; asterisk, lip binding cysteine; underlined letters, amino acids determined by Edman degradation sequencing; red letters, amino acid sequences determined by liquid chromatography-MS/MS; double underlined blue letters, amino acids determined by MALDI-TOF MS). B, the SitC protein fragments obtained by lysylendopeptidase digestion in an SDS-polyacrylamide gel slice were measured by MALDI-TOF MS. A series of mass peaks harboring 14 mass differences were numbered 1–8 in the left panel and described in the right panel. The obtained m/z of peak 1 corresponded to theoretical m/z of tripalmitic acid (Pam3)-modified N-acyl-S-(diacyl-propyl)-cysteinyl-peptide, and those of peaks 2–8 corresponded with Pam3-N-terminal peptide harboring increasing numbers of methylene (CH2) groups in their fatty acids. C and D, CHO/hCD14/hTLR2 cells (C) or CHO/hCD14/hTLR4 (D) were stimulated in the absence (black line with gray area) or in the presence of 2 ng/ml (blue) or 20 ng/ml (magenta) of the purified S. aureus SitC protein. Surface expression of NF-κB-driven hCD25 was analyzed by flow cytometry.

**TABLE**

| Position | m/z Obtained | Corresponding Structure & its m/z |
|----------|--------------|----------------------------------|
| 1        | 1310.9798    | Pam3-GCTGGK, 1310.9604           |
| 2        | 1324.9886    | (Pam3+CH2)-GCTGGK, 1324.9760     |
| 3        | 1338.9963    | Pam3+(CH2)2-GCTGGK, 1338.9917   |
| 4        | 1353.0136    | Pam3+(CH2)3-GCTGGK, 1353.0073   |
| 5        | 1367.0318    | Pam3+(CH2)4-GCTGGK, 1367.0230   |
| 6        | 1381.0498    | Pam3+(CH2)5-GCTGGK, 1381.0386   |
| 7        | 1395.0704    | Pam3+(CH2)6-GCTGGK, 1395.0543   |
| 8        | 1409.0792    | Pam3+(CH2)7-GCTGGK, 1409.0699   |

The 33-kDa protein is identified as triacylated SitC lipoprotein. A, indicates the whole amino acid sequence of the S. aureus SitC protein (box, signal peptide; asterisk, lip binding cysteine; underlined letters, amino acids determined by Edman degradation sequencing; red letters, amino acid sequences determined by liquid chromatography-MS/MS; double underlined blue letters, amino acids determined by MALDI-TOF MS). B, the SitC protein fragments obtained by lysylendopeptidase digestion in an SDS-polyacrylamide gel slice were measured by MALDI-TOF MS. A series of mass peaks harboring 14 mass differences were numbered 1–8 in the left panel and described in the right panel. The obtained m/z of peak 1 corresponded to theoretical m/z of tripalmitic acid (Pam3)-modified N-acyl-S-(diacyl-propyl)-cysteinyl-peptide, and those of peaks 2–8 corresponded with Pam3-N-terminal peptide harboring increasing numbers of methylene (CH2) groups in their fatty acids. C and D, CHO/hCD14/hTLR2 cells (C) or CHO/hCD14/hTLR4 (D) were stimulated in the absence (black line with gray area) or in the presence of 2 ng/ml (blue) or 20 ng/ml (magenta) of the purified S. aureus SitC protein. Surface expression of NF-κB-driven hCD25 was analyzed by flow cytometry.
isk in Fig. 2A, respectively. SitC has been suggested to be anchored to the outer leaflet of the cytoplasmic membrane by its lipid-modified N terminus and also to localize in the cell wall layer by studies using immunoelectron microscopic analysis of *Staphylococcus epidermidis* (23). These previous results are consistent with our current observations that SitC was triacylated lipoprotein and recovered from the insoluble PGN. In addition, SitC is known to be an iron-regulated ATP binding cluster (ABC) transporter substrate-binding protein (23) and is known to function as one of the major lipoproteins in *S. aureus* (24).

The purified SitC protein was capable of stimulating TLR2 but not TLR4 at a concentration of 20 ng/ml (0.6 nM) (Fig. 2, C and D). This concentration is comparable with that of lipopolysaccharide, which can induce the production of cytokines in the mouse macrophage. Taken together, these results suggest that the triacylated SitC protein released from the insoluble *S. aureus* PGN by *Achromobacter*/H9252-lytic protease treatment functions as a native ligand of TLR2 in vitro.

**Cytokine Production by SitC Requires TLR2 and MyD88**—We next evaluated the cytokine production ability of the purified SitC lipoprotein using mice thioglycolate-elicited peritoneal macrophages and found that the SitC protein induced the release of tumor necrosis factor-α and interleukin-6 at a concentration of 100 ng/ml (3 nM) from wild-type mouse macrophages (Fig. 3, A and B). We further tested which TLR is required for the SitC-mediated production of cytokine, since TLR2 has been thought to function as a heterodimer with TLR1 or TLR6. As expected, the release of tumor necrosis factor-α and interleukin-6 from TLR2*/−*/H11002/TLR1*/−*/H11002 mouse macrophages was not induced by SitC (Fig. 3, A and B). Unexpect-
ABSTRACT

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SitC-deficient S. aureus Cells Still Retain TLR2 Stimulation Activity—In order to examine whether or not the S. aureus SitC protein is truly a dominant ligand for TLR2 stimulation, we generated an S. aureus sitC deletion mutant (ΔsitC), which was viable in Luria-Bertani medium and tetracyclase broth. Further examination of the 33-kDa protein in the Triton X-114 detergent phase of the solubilized PGN fraction revealed that, as expected, the SitC 33-kDa protein band was not detected in ΔsitC mutant and was recovered by introducing a plasmid harboring the sitC gene into ΔsitC (Fig. 3C, lanes 2 and 4, respectively), demonstrating that the 33-kDa protein in the PGN layer is the bona fide SitC protein. We subsequently tested how the sitC mutation affected TLR2 stimulation activity. When we compared TLR2 stimulation activity from the cells of the S. aureus parent and ΔsitC mutant strains on TLR2-expressing CHO cells, the ΔsitC mutant cells showed approximately the same TLR2 stimulation activity as the cells from the parent strain (Fig. 3, D and E). In addition, no reduction was observed in TLR2-mediated NF-κB promoter activity with ΔsitC mutant on the TLR2-expressing HEK293 cells (Fig. S1C). These results suggest that the SitC lipoprotein is not a predominant TLR2 ligand in S. aureus itself, indicating that other lipoproteins are also capable of activating TLR2.

Two Additional ABC Transporter Substrate-binding Lipoproteins from Gram-positive Bacteria Also Activate TLR2—We hypothesized that the ABC transporter substrate-binding lipoproteins from other Gram-positive bacteria may also function as natural ligands for TLR2. In order to test this notion, we purified two different Triton X-114-enriched proteins to homogeneity from Bacillus subtilis (33 kDa) and Micrococcus luteus (37 kDa) (Fig. 4A, lanes 1 and 2, respectively). When we examined whether or not these two purified proteins are capable of stimulating TLR2 in TLR2-expressing CHO cells, the two proteins clearly showed TLR2 stimulation activity (Fig. 4, B and D) but not on TLR4 (Fig. 4, C and E) at a concentration of 20 ng/ml. The liquid chromatography-MS/MS analyses of fragments obtained by trypsin treatment indicated that the 33- and 37-kDa proteins can also be assigned as tentative ABC transporter substrate-binding lipoproteins (NP_388633 and ZP_02945718 in B. subtilis and M. luteus, respectively; Fig. S2). The B. subtilis lipoprotein (33 kDa) is encoded by the yfmC gene and produces a 315-amino acid precursor harboring the putative lipid binding cysteine at amino acid position 19, which is similar to the ferrichrome-binding protein of the ABC transporter. In addition, the M. luteus protein (37 kDa) is encoded by a gene producing a 297-amino acid precursor harboring the putative lipid binding cysteine at the amino acid position 22, which is analogous to GluB (glutamate-binding protein) of the ABC transporter system. These results strongly demonstrate that the ABC transporter substrate-binding lipoproteins from Gram-positive bacteria are native ligands of TLR2.

DISCUSSION

Here, we report for the first time that three ABC transporter substrate-binding proteins from three different Gram-positive bacteria function as native TLR2 ligands in vitro. In addition, we show that the LTA and the PGN of S. aureus is not a predominant ligand for TLR2. Initially, the 33-kDa SitC protein from S. aureus was reported to be one of the major lipoproteins distributed within the cell wall and to be induced under conditions where iron was restricted (23), indicating that the SitC protein is induced in the host after bacterial infection because the concentration of free iron in tissue has been estimated to be as low as 10⁻¹⁲ M (25). In addition, about 40% of the lipoprotein genes in S. aureus encode for substrate-binding proteins (22 of 49 putative lipoprotein genes in strain N315; data not shown). In addition, the primary domain structures of these substrate-binding proteins are only observed in bacteria species, but not in mammalian. Thus, taking these reported data and our current data into consideration, it would be reasonable to conclude that the pattern recognition receptor TLR2 senses the abundant bacterial ABC transporter substrate-binding lipoproteins as pathogen-associated molecular patterns that are either expressed on the Gram-positive bacterial cell surface or secreted into the extracellular space during the innate immune responses.

Moreover, this study first provides biochemical evidence that staphylococcal lipoproteins are triacylated. This is a surprising result because, until now, S. aureus lipoproteins are considered to be diacylated due to the absence of Lnt protein, which adds a fatty acid to the amino group of S-(diacyl-propyl) cysteine. Thus, S. aureus must have another type of N-acyltransferase.

FIGURE 4. ABC transporter substrate-binding lipoproteins of two another Gram-positive bacteria activate TLR2. A, SDS-PAGE analysis of purified lipoproteins of S. aureus SitC (lane SitC), B. subtilis YfmC (lane 1), and M. luteus GluB (lane 2). B–E, CHO/hCD14/hTLR2 (B and D) or CHO/hCD14/hTLR4 (C and E) cells were stimulated in the absence (black line with gray area) or in the presence of 2 ng/ml (blue) or 20 ng/ml (magenta) of the B. subtilis YfmC lipoprotein (B and C) or of the M. luteus GluB lipoprotein (D and E). The experiments were performed as described in the legend to Fig. 1 and were representative of at least two independent experiments.
whose structure is distinct from the E. coli Lnt protein. We further demonstrated that the SitC-mediated cytokine production required TLR2 but not TLR6 or TLR1 in the studies using peritoneal macrophages of TLR KO mice. This finding indicates that the SitC lipoprotein can signal either in a TLR1- and TLR6-independent manner or, alternatively, by the TLR2/TLR1 or TLR2/TLR6 heterodimer, since the TLR2 homodimer is not active and TLR2 needs to pair with either TLR1 or TLR6 in order to induce signaling (26). Although this elucidation is in disagreement with the hypothesis that the triacylated lipoproteins only signal via TLR1/TLR2 (27, 28), our data were consistent with other recent studies that have demonstrated that some synthetic di- and triacylated lipopeptides signal in TLR1-deficient cells, in TLR6-deficient cells, and in human epithelial cell lines transfected with TLR2/TLR1 or TLR2/TLR6 (29–31). Taken together, the present study identified ABC transporter substrate-binding lipoproteins as native TLR2 lipids from Gram-positive bacteria and reveals that the triacylated SitC lipoprotein can signal either in a TLR1- and TLR2-independent manner or, alternatively, by the TLR2/TLR6 heterodimer, since the TLR2 homodimer is not active and TLR2 needs to pair with either TLR1 or TLR6 in order to induce signaling (26). Although this elucidation is in disagreement with the hypothesis that the triacylated lipoproteins only signal via TLR1/TLR2 (27, 28), our data were consistent with other recent studies that have demonstrated that some synthetic di- and triacylated lipopeptides signal in TLR1-deficient cells, in TLR6-deficient cells, and in human epithelial cell lines transfected with TLR2/TLR1 or TLR2/TLR6 (29–31). Taken together, the present study identified ABC transporter substrate-binding lipoproteins as native TLR2 lipids from Gram-positive bacteria and reveals that the triacylated S. aureus SitC lipoprotein signals via TLR2/TLR1 or TLR2/TLR6 in murine peritoneal macrophages.

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