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*J Immunol* published online 9 November 2011
http://www.jimmunol.org/content/early/2011/11/09/jimmunol.1100499

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A Novel Bacterial Resistance Mechanism against Human Group IIA-Secreted Phospholipase A2: Role of Streptococcus pyogenes Sortase A

Elin Movert,* Yongzheng Wu,†‡ Gérard Lambeau,§ Lhoussine Touqui,†‡ and Thomas Areschoug* 

Human group IIA-secreted phospholipase A2 (sPLA2-IIA) is a bactericidal molecule important for the innate immune defense against Gram-positive bacteria. In this study, we analyzed its role in the host defense against Streptococcus pyogenes, a major human pathogen, and demonstrated that this bacterium has evolved a previously unidentified mechanism to resist killing by sPLA2-IIA. Analysis of a set of clinical isolates demonstrated that an ~500-fold higher concentration of sPLA2-IIA was required to kill S. pyogenes compared with strains of the group B Streptococcus, which previously were shown to be sensitive to sPLA2-IIA, indicating that S. pyogenes exhibits a high degree of resistance to sPLA2-IIA. We found that an S. pyogenes mutant lacking sortase A, a transpeptidase responsible for anchoring LPXTG proteins to the cell wall in Gram-positive bacteria, was significantly more sensitive (~30-fold) to sPLA2-IIA compared with the parental strain, indicating that one or more LPXTG surface proteins protect S. pyogenes against sPLA2-IIA. Importantly, using transgenic mice expressing human sPLA2-IIA, we showed that the sortase A-mediated sPLA2-IIA resistance mechanism in S. pyogenes also occurs in vivo. Moreover, in this mouse model, we also showed that human sPLA2-IIA is important for the defense against lethal S. pyogenes infection. Thus, we demonstrated a novel mechanism by which a pathogenic bacterium can evade the bacterial action of sPLA2-IIA and we showed that sPLA2-IIA contributes to the host defense against S. pyogenes infection. The Journal of Immunology, 2011, 187: 000–000.

When a pathogenic bacterium enters the human body, it is challenged with a plethora of antimicrobial molecules, including secreted phospholipases A2 (sPLA2s), antimicrobial peptides (AMPs), and chemokines, which directly kill the invading microbe by various mechanisms (1, 2). Some of these antimicrobial molecules are constitutively expressed in human tissues, but most are induced and secreted by many cell types during inflammation. To be able to survive in this hostile environment and establish an infection, a pathogenic bacterium must evolve mechanisms to resist killing by these antimicrobial molecules (1, 2).

Human sPLA2s form a relatively small family of structurally related enzymes that hydrolyze the sn2 bond of phospholipids, generating lysophospholipids and free fatty acids (3–5). One member of this family, the human group IIA sPLA2 (sPLA2-IIA), is constitutively expressed by, for example, Paneth cells, and its expression can be induced upon infection in a TLR-dependent manner in several cell types, including macrophages, giving rise to high concentrations of sPLA2-IIA in plasma and in the mucosa (2, 6). A growing body of evidence suggests that sPLA2-IIA represents an important part of the innate immune defense against bacterial pathogens. In vitro analysis using purified sPLA2-IIA demonstrated that this enzyme shows direct bactericidal activity against a wide range of bacterial pathogens, in particular Gram-positive bacteria (7–13). Moreover, studies using naturally sPLA2-IIA-deficient mice or transgenic (Tg) mice overexpressing human sPLA2-IIA demonstrated an important protective role for sPLA2-IIA against experimental infection with several bacterial pathogens, including Staphylococcus aureus, Escherichia coli, and Bacillus anthracis (14–17).

In Gram-positive bacteria, it is believed that the positively charged sPLA2-IIA molecule initially is attracted to negatively charged surface moieties of the bacteria, such as wall teichoic acid and lipoteichoic acid (LTA), after which it penetrates the cell wall peptidoglycan and eventually reaches the cellular membrane, where sPLA2-IIA hydrolyzes the phospholipids in a Ca2+-dependent manner (18, 19). To resist killing by sPLA2-IIA, Gram-positive bacteria have been shown to modify their LTA with D-alanylation, which lower the net negative charge of the LTA polymer and are believed to inhibit the ability of sPLA2-IIA to cross the cell wall (18, 20). D-alanylation of LTA was similarly shown to be involved in the resistance to killing by positively charged AMPs, including the human cathelicidin LL-37 (21, 22), indicating that it is a general mechanism among Gram-positive bacteria to resist positively charged antibacterial molecules. However, although most Gram-positive bacteria express D-alany-
lated LTA (23), the sensitivity to sPLA2-IIA differs greatly between species (11, 12), indicating that some Gram-positive pathogens may have evolved other resistance mechanisms to sPLA2-IIA. In this study, we identified a novel bacterial molecule, the transpeptidase sortase A (SrtA) of *Streptococcus pyogenes*, that is involved in the resistance to sPLA2-IIA. *S. pyogenes* is a major Gram-positive pathogen causing a wide range of clinical manifestations in humans, such as pharyngitis, skin infections, and a toxic shock syndrome. It also gives rise to two important post-infectious sequelae: acute rheumatic fever and acute glomerulonephritis (24). *S. pyogenes* expresses several cell surface proteins that are important for various steps in the molecular pathogenesis of *S. pyogenes* infection, such as phagocytosis resistance, adhesion, and invasion of host cells (25, 26). Many of these cell surface proteins, including the antiphagocytic M protein, belong to a class of proteins containing a C-terminal LPXTG motif that are anchored to the bacterial cell wall peptidoglycan through a process that involves the transpeptidase SrtA (27, 28). SrtA recognizes the LPXTG motif and catalyzes the transpeptidation reaction that results in covalent binding of the protein to peptides in cell wall peptidoglycan (27, 28). Studies on Gram-positive pathogens showed that SrtA mutants are significantly less virulent compared with their wild-type (WT) counterparts in animal models (29–32), demonstrating that LPXTG proteins, in general, are important virulence factors.

In this article, we show that clinical strains of *S. pyogenes* exhibit a high degree of resistance against sPLA2-IIA. In an attempt to identify the bacterial factors that contribute to this resistance, we found that SrtA is important for *S. pyogenes* to resist direct killing by sPLA2-IIA, indicating that one or more LPXTG surface proteins are involved in this innate immune-evasion mechanism. Moreover, using Tg mice expressing human sPLA2-IIA, we present evidence that this resistance mechanism also occurs in vivo and that sPLA2-IIA contributes to the innate immune defense against lethal *S. pyogenes* infection. Thus, we demonstrate a novel mechanism by which a pathogenic bacterium can evade the direct bactericidal action of sPLA2-IIA and, to our knowledge, we show for the first time that sPLA2-IIA contributes to the host defense against *S. pyogenes* infection.

**Materials and Methods**

**Bacterial strains**

The WT *S. pyogenes* serotype M6 strain JRS8, the isogenic SrtA mutant strain (JRS758), and the sortase B (SrtB)-mutant strain (JRS789) were constructed as described (33) and kindly provided by Dr. J.R. Scott (Emory University School of Medicine, Atlanta, GA). Plasmid pJRS757, containing the intact srtA gene, was constructed as described (33) and used for trans-complementation of the SrtA mutant. A collection of clinical *S. pyogenes* strains representing serotypes IA, IIB, M5, M18, M22, and M28 and the group B *Streptococcus* (GBS) strains A909, BM110, NEM316, and 1084, representing the clinically important serotypes Ia, III, III, and V, respectively, were kindly provided by Dr. G. Lindahl (Lund University). Strains of *S. pyogenes* were grown in Todd–Hewitt broth supplemented with 0.2% yeast extract (THY) in 5% CO2 at 37°C and GBS strains were grown in Todd–Hewitt broth at 37°C.

**Proteins and reagents**

Recombinant WT human sPLA2-IIA was produced in *Drosophila* S2 cells using a protocol similar to that previously used for production in baculovirus-infected insect cells (34) and following the use in insect cells for the structurally related human group IID sPLA2 (35). The sPLA2-IIA preparation was checked for purity by SDS-PAGE analysis, MALDI-TOF mass spectrometry, and sPLA2-IIA enzymatic assays (35). Before use, the purified lyophilized sPLA2-IIA preparation was solubilized in PBS. The sPLA2-IIA-specific inhibitory antibody LY311727 was described previously (36). The human AMP LL-37 and Magainin II were purchased from Sigma (St. Louis, MO).

**Mice**

Female human sPLA2-IIA Tg mice (C57BL/6N-Tg[N(sPLA2)]) and C57BL/6 WT mice were maintained, as described (37). The WT C57BL/6 mice have a natural mutation in the gene encoding mouse sPLA2-IIA (*Pla2g2a*); therefore, they are deficient for the mouse enzyme (38). In the Tg mouse model, the transgene fragment contains the entire human sPLA2-IIA gene together with a 1.6-kb region upstream of the sPLA2-IIA gene, with the promoter and response elements for diverse effectors (IL-6, IFN, hepatocyte NF-3, AP1, AP2, C/EBP, and cAMP response element) (37). All animal experiments were performed in strict accordance with guidelines from the Lund/Malmö ethical review board on animal experiments.

**Bactericidal analysis with recombinant human sPLA2-IIA and AMPs**

The sPLA2-IIA bactericidal assay was adapted from a previously described protocol (8, 9). Briefly, overnight cultures of streptococci (OD620 = 1.1) were diluted 20-fold into fresh broth, and bacteria were grown at 37°C to mid-log phase (OD620 = 0.4). Bacteria were then directly diluted 1000-fold into HEPES buffer (20 mM HEPES, 2 mM Ca2+, 1% BSA [pH 7.4]) to give a bacterial concentration of ∼1 × 107 CFU/mL. The recombinant human sPLA2-IIA or AMP was serially diluted in HEPES buffer, and small aliquots of 10 µl were added in triplicates to the wells of a sterile 96-well polystyrene plate (Corning) after which it was mixed with 10 µl the bacterial suspension. After incubation at 37°C with 5% CO2 for 2 h, the samples were serially diluted in PBS, plated onto blood agar plates, and incubated overnight to determine the bacterial viability by colony counting. In the inhibition experiment, LY311727 was added at a final concentration of 50 µM. In the analysis of the role of bacterial growth phase on sensitivity to sPLA2-IIA, bacteria were used at mid-log phase (OD620 = 0.4) and early stationary phase (OD620 = 0.9).

**Radiolabeling of *S. pyogenes* membrane and analysis of bacterial phospholipid degradation**

Bacterial phospholipids were labeled, essentially as described (8). Briefly, overnight cultures of streptococci were inoculated into fresh THY containing 1 µCi [3H]oleic acid ([3H]-OA) and grown to mid-log phase (OD620 = 0.4). After centrifugation, the bacterial pellet was resuspended in fresh THY and incubated for an additional 30 min at 37°C. After washing three times in 0.15 M NaCl to remove free oleic acid, the bacteria were resuspended in HEPES buffer (20 mM [pH 7.4]) containing 1% fatty acid-free BSA and used promptly. The bacteria were incubated with 2.5 µg/mL sPLA2-IIA for 2 h at 37°C. In the inhibition experiment using LY311727, the sPLA2-IIA dilution was preincubated with 50 µM LY311727 for 30 min at 37°C before incubation with bacteria. Bacterial phospholipid degradation products generated by sPLA2-IIA treatment were quantitatively recovered from centrifuged extracellular medium as complexes with BSA and measured by liquid scintillation spectroscopy. Phospholipid degradation was calculated as a percentage of total sample radioactivity.

**sPLA2-IIA assays**

Tissue homogenates were prepared, as previously described (6). Frozen tissues were suspended in 10 volumes of a lysis buffer containing 0.25 mM sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.5 mM PMSF, 2 µg/mL leupeptin, and 2 µg/mL aprotinin and disrupted with an Ultra-Turrax T-25 (Janke and Kunkel) at 4°C. The homogenates were centrifuged for 5 min at 1,000 × g, the soluble fractions were collected and centrifuged for 20 min at 20,000 × g, and the resulting supernatant was used to evaluate sPLA2-IIA activity. sPLA2-IIA activity was assayed using [3H]oleate-labeled membranes of *E. coli*, following a modification of the method of Fran-son et al. (39). The identity and level of the sPLA2-IIA protein in tissue homogenates and body fluids from Tg sPLA2-IIA and corresponding littermate mice were determined by inhibition of sPLA2-IIA activity with the inhibitor LY311727, as previously described (17).
bacterial pellet was measured with a gamma counter. In another binding assay, mid-log–phase bacteria were washed in HEPES buffer and suspended to a 1% suspension (~1 × 10^6 CFU/ml). Bacteria were mixed with various amounts of [125I]sPLA2-IIA (50,000, 10,000 and 2,000 cpm) and incubated at 37°C for 10 min. The bacteria were washed twice in PBS, boiled in sample buffer for 5 min, and subjected to SDS-PAGE, after which proteins were blotted onto a nitrocellulose membrane and analyzed by autoradiography.

To verify that sPLA2-IIA was bound to the surface of bacteria, mid-log–phase JRS4 bacteria were washed in HEPES buffer and diluted to ~1 × 10^9 CFU/ml. Bacteria were then mixed with [125I]sPLA2-IIA (~35,000 cpm). After incubation at RT for 10 min, bacteria were washed twice with PBS, and radioactivity associated with the bacterial pellet was measured with a gamma counter. The [125I]sPLA2-IIA bound to JRS4 was then eluted with 1 M NaCl at RT for 30 min. The bacterial suspension was washed three times in PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter.

To analyze sPLA2-IIA bactericidal activity after incubation with bacteria, the WT strain JRS4 was grown to mid-log phase (OD_{600} = 0.4). JRS4 bacteria were washed once in HEPES buffer and incubated with 100 μl sPLA2-IIA (final concentration, 12.5 μg/ml) for 10 min at 37°C. The bacteria were centrifuged, and the supernatant (containing sPLA2) was diluted 10×, 100×, and 500× in HEPES buffer, and a killing assay analyzing the bactericidal activity in the eluates against GBS strain BM110 (grown to mid-log phase) was performed. Aliquots of diluted eluates were added in triplicates to a sterile 96-well polypropylene plate and mixed with the bacterial suspension (~1 × 10^9 CFU/ml). After incubation at 37°C for 30 min, the samples were diluted in PBS and plated onto blood agar plates to determine the bacterial viability by colony counting. The inhibitor LY311727 (50 μM) was used in control wells to verify that the bactericidal activity was specific for sPLA2-IIA in the eluates.

**Mouse experiments**

Groups of WT C57BL/6 or Tg C57BL/6NTac-TgN (human sPLA2-IIA) mice were infected i.p. with a 1:1 mixture of mid-log–phase JRS4 WT and SrtA mutant bacteria. This mixture was defined as the input. Each mouse received ~5 × 10^8 CFU. Surviving mice were sacrificed after 18 h, and spleens and livers were collected. Organs from a total of 11 WT C57BL/6 and 10 Tg C57BL/6NTac-TgN (human sPLA2-IIA) mice were analyzed. The organs were homogenized, serially diluted in PBS, and plated on blood agar plates. The bacteria in these samples, defined as the output, were analyzed for the presence of the two strains in the input. For each organ, a total of 100–200 colonies were picked and grown in short patches on THY agar containing 100 μg/ml spectinomycin. On these plates, only the SrtA mutant strain grows because it contains a spectinomycin resistance gene. To analyze the role of sPLA2-IIA in the protection against lethal S. pyogenes infection, the WT strain JRS4 was grown to mid-log phase and diluted in ice-cold THY. Groups of six C57BL/6 and Tg C57BL/6NTac-TgN (sPLA2-IIA) mice were infected i.p. with ~8 × 10^8 CFU of JRS4 WT bacteria, representing an ~90% lethal dose in the C57BL/6 mice. Mice were checked every 4 h, and deaths were recorded daily.

**Statistical analysis**

Cs derived from mixed-infection experiments were analyzed with the Student t test. The significance in difference in lethality between WT C57BL/6 and Tg sPLA2-IIA mice when infected with S. pyogenes strain JRS4 was calculated with the two-tailed Fisher exact test.

**Results**

**Clinical strains of S. pyogenes show a high degree of resistance against the bacterial activity of human sPLA2-IIA**

We analyzed bactericidal activity of human sPLA2-IIA to S. pyogenes in comparison with GBS, a major neonatal pathogen (42) previously shown to be relatively sensitive to direct killing by sPLA2-IIA (12). We selected a number of clinical S. pyogenes isolates representing several important M serotypes (M1, M4, M5, M6, M18, M22, and M28) and GBS strains representing the three clinically most important capsular serotypes (Ia, III, and V). As shown in Fig. 1, this analysis demonstrated striking differences between S. pyogenes and GBS with regard to sensitivity to the bactericidal activity of human sPLA2-IIA. For GBS, the concentration of sPLA2-IIA killing 50% of the bacteria ranged from ~0.5 to ~4 ng/ml, whereas it ranged from ~0.1 to ~2 μg/ml for S. pyogenes. Thus, on average, a ~500-fold higher concentration of sPLA2-IIA is required to kill S. pyogenes compared with GBS, demonstrating that strains of S. pyogenes exhibit a high degree of resistance to sPLA2-IIA.

S. pyogenes SrtA contributes to the resistance against human sPLA2-IIA

As described above, α-analyses of surface LTA was described as a major resistance mechanism to human sPLA2-IIA in Gram-positive bacteria (18, 20). However, because both S. pyogenes and GBS express α-analylated LTA (43, 44), we hypothesized that strains of S. pyogenes may have developed other mechanisms to resist direct killing by sPLA2-IIA, and we focused on investigating the role of surface proteins in this context. To investigate this hypothesis, we analyzed two isogenic S. pyogenes mutants, lacking either of the sortases SrtA or SrtB, derived from the M6 strain JRS4, in our bactericidal assay. SrtA was shown to be responsible for the covalent surface attachment of several well-known LPXTG proteins in S. pyogenes implicated as virulence factors, such as the M protein, the C5a peptidase, protein F, and protein GRAB (33), whereas SrtB was shown to be responsible for cell wall anchoring of the T6 protein, which is a subunit of pili in JRS4 (33). As demonstrated in Fig. 2A, the SrtA mutant, but not the SrtB mutant, is significantly more sensitive to the bactericidal activity of human sPLA2-IIA compared with JRS4. Indeed, a ~30-fold lower concentration of sPLA2-IIA was enough to kill 50% of the SrtA mutant compared with the WT strain JRS4. To exclude that this difference is due to a polar effect or secondary mutation in the isogenic SrtA mutant, we performed trans-complementation analysis by transforming the SrtA mutant with plasmid pJR577, which contains the intact srtA gene. Trans-complementation of SrtA with pJR577 was previously demonstrated to restore the WT phenotype in the SrtA mutant (33). As shown in Fig. 2B, the trans-complemented strain SrtA/pJR577 was equally resistant to sPLA2-IIA as the WT strain JRS4, showing that the sensitivity of the SrtA mutant to sPLA2-IIA is completely due to the lack of srtA. This result indicated that one or more LPXTG surface proteins contribute to the protection against sPLA2-IIA attack in S. pyogenes, demonstrating a novel bacterial-resistance mechanism against this bacterial molecule. Importantly, killing of the SrtA mutant was mediated by sPLA2-IIA alone, because the bactericidal activity was strongly inhibited by the sPLA2-IIA inhibitor LY311727 (Fig. 2C). In an attempt to identify the LPXTG protein in S. pyogenes involved in the resistance to sPLA2-IIA, we analyzed the sensitivity of isogenic S. pyogenes mutants lacking the M6 protein and the fibronectin-binding protein F, but they were equally resistant to sPLA2-IIA as the parental strain JRS4 (data not shown).

To analyze the killing kinetics, we chose a concentration of sPLA2-IIA (2.5 μg/ml; Fig. 2A) at which the SrtA mutant bacteria are completely killed and the WT strain JRS4 bacteria are only partially killed after 2 h. Strikingly, most of the SrtA mutant bacteria were killed within 10 min (Fig. 3A) at this concentration, whereas the killing of WT strain JRS4 was much slower. Thus, SrtA also contributes to the resistance against sPLA2-IIA by slowing down the killing rate of S. pyogenes.

It was previously shown that growing Gram-positive bacteria, but not stationary-phase bacteria, are sensitive to sPLA2-IIA, implying that sites in the bacterial envelope engaged in cell
growth represent an important target of attack for sPLA2-IIA (45).

To investigate whether this was also the case in our system, we compared the sensitivity of the JRS4 WT and SrtA- mutant bacteria to sPLA2-IIA in early stationary phase (OD620 = 0.9) and mid-log phase (OD620 = 0.4). As shown above, when analyzed in mid-log phase, the SrtA- mutant was sensitive to sPLA2-IIA; however, when it was analyzed in stationary phase, the SrtA- mutant was equally as resistant to sPLA2-IIA as JRS4 (Fig. 3B).

**FIGURE 1.** Bactericidal activity of sPLA2-IIA against clinical strains of *S. pyogenes* and GBS. Overnight cultures of streptococci were diluted 20 times into fresh broth, and bacteria were grown to mid-log phase (OD620 = 0.4). Bacteria were directly diluted 1000-fold into HEPES buffer (20 mM HEPES, 2 mM Ca2+, 1% BSA [pH 7.4]) to give a bacterial concentration of ~1 × 10^5 CFU/ml. Recombinant human sPLA2-IIA was serially diluted in HEPES buffer, and small aliquots of 10 μl were added in triplicates to sterile 96-well polypolyyprolene plates, after which it was mixed with 10 μl of the bacterial suspension. After incubation at 37°C in 5% CO2 for 2 h, the samples were diluted in PBS, plated onto blood agar plates, and incubated overnight to determine the bacterial viability by colony counting. The killing is presented as the percentage of CFU for each bacterial strain remaining in the wells after incubation with sPLA2-IIA compared with the inoculum.

**FIGURE 2.** SrtA significantly contributes to the ability of *S. pyogenes* to resist the bactericidal activity of sPLA2-IIA. A, Overnight cultures of the WT strain JRS4 and the isogenic SrtA- mutant were diluted 20 times into fresh broth, and bacteria were grown to mid-log phase (OD620 = 0.4). Bacteria were directly diluted 1000-fold into HEPES buffer to give a bacterial concentration of ~1 × 10^5 CFU/ml. sPLA2-IIA was serially diluted in HEPES buffer, and small aliquots of 10 μl were added in triplicates to sterile 96-well polypolyyprolene plates, after which it was mixed with 10 μl of the bacterial suspension. After incubation at 37°C in 5% CO2 for 2 h, the samples were diluted in PBS, plated onto blood agar plates, and incubated overnight to determine the bacterial viability by colony counting. The killing is presented as the percentage of CFU for each bacterial strain remaining in the wells after incubation with sPLA2-IIA compared with the inoculum. B, *Trans*-complementation analysis where the SrtA- mutant strain was transformed with plasmid pJRS757 containing the intact srtA gene. The resulting *trans*-complemented strain (SrtA-/pJRS757) was compared with the WT strain JRS4 and the SrtA- mutant in sensitivity to sPLA2-IIA using the protocol described above. C, The experiment was essentially performed as described in A, but the sPLA2-IIA inhibitor LY311727 was added to a final concentration of 50 μM.
FIGURE 3. Analysis of the sPLA₂-IIA–killing kinetics and role of bacterial growth phase of the WT JRS4 strain and the SrtA mutant. A, Overnight cultures of the WT strain JRS4 and the isogenic SrtA– mutant were diluted 20 times into fresh broth, and bacteria were grown to mid-log phase (OD₆₂₀ = 0.4). Bacteria were directly diluted 500-fold into HEPES buffer to give a bacterial concentration of ~2 × 1⁰⁷ CFU/ml. sPLA₂-IIA was diluted in HEPES buffer (to give a final concentration of 2.5 μg/ml), and small aliquots of 10 μl were added in triplicates to sterile 96-well polypropylene plates, after which it was mixed with 10 μl of the bacterial suspension. The wells were incubated at 37°C in 5% CO₂, and samples were diluted in PBS at various time points, plated onto blood agar plates, and incubated overnight to determine the bacterial viability by colony counting. The killing is presented as the percentage of CFU for each bacterial strain remaining in the wells after incubation with sPLA₂-IIA compared with the inoculum. B, The WT JRS4 and SrtA– mutant strains were grown to mid-log phase (OD₆₂₀ = 0.4; log) and early stationary phase (OD₆₂₀ = 0.9; stat) and compared for sensitivity to sPLA₂-IIA.

This result showed that sPLA₂-IIA is bactericidal only against growing bacteria and that SrtA most likely mediates protection of S. pyogenes against sPLA₂-IIA at sites in the bacterial envelope engaged in growth.

SrtA is necessary for S. pyogenes to resist sPLA₂-IIA–mediated degradation of membrane phospholipids

The bactericidal activity of sPLA₂-IIA is due to its ability to degrade the bacterial membrane phospholipids. Thus, we examined the effect of sPLA₂-IIA on [³H]-OA release from [³H]-OA–labeled JRS4 WT and SrtA– mutant bacteria in the bactericidal assay. As shown in Fig. 4, ~14% of the total [³H]-OA was released from the SrtA– mutant, whereas only ~2.5% of the total [³H]-OA was released from the WT JRS4 strain. Importantly, release of [³H]-OA from the SrtA– mutant was completely inhibited by LY311727, which inhibits the enzymatic activity of sPLA₂-IIA, confirming that [³H]-OA release from the SrtA– mutant is due to degradation of phospholipids by sPLA₂-IIA. This result showed that sPLA₂-IIA degrades the membrane phospholipids to a greater extent in the SrtA– mutant compared with the parental strain JRS4, which clearly correlates with the bactericidal activity of sPLA₂-IIA against the SrtA– mutant. Thus, SrtA protects S. pyogenes against degradation of the cellular membrane phospholipids by sPLA₂-IIA.

Human sPLA₂-IIA shows greater binding to S. pyogenes WT bacteria compared with the SrtA– mutant

Our data above showed that SrtA protects S. pyogenes from killing by sPLA₂-IIA, indicating that one or more S. pyogenes LPXTG proteins are involved in the protection against this bactericidal molecule. We hypothesized that an LPXTG protein(s) contributes to the binding of sPLA₂-IIA to S. pyogenes by binding and sequestering sPLA₂-IIA at the bacterial surface, thereby preventing it from reaching the cellular phospholipid membrane. Assuming that this hypothesis is correct, the WT JRS4 bacteria may show greater binding to sPLA₂-IIA compared with the SrtA– mutant. To test this hypothesis, we analyzed the binding of radio iodinated sPLA₂-IIA to JRS4 and the SrtA– mutant. As shown in Fig. 5A and 5B, sPLA₂-IIA bound more avidly to the WT JRS4 bacteria compared with the SrtA– mutant. To exclude the possibility that the greater amount of sPLA₂-IIA bound to JRS4 compared with the SrtA– mutant was due to differences in internalization of sPLA₂-IIA rather than direct cell surface binding, we performed sPLA₂-IIA–elution experiments with high ionic strength, a washing step that is known to release bound sPLA₂-IIA from anionic components present at cellular surfaces (46). We incubated JRS4 with sPLA₂-IIA, washed the bacteria, and subsequently eluted surface-bound sPLA₂-IIA with 1 M NaCl. As shown in Fig. 5C, <10% of the bound sPLA₂-IIA remained associated with JRS4 after incubation in 1 M NaCl, showing that almost all sPLA₂-IIA was bound to the surface.

sPLA₂-IIA incubated with WT JRS4 bacteria retains its bactericidal properties

S. pyogenes possesses several LPXTG surface proteins that act as proteases, including the C5a peptidase and SpyCEP, which cleave and inactivate host defense molecules, such as complement C5a...
was performed. Aliquots of diluted sPLA2-IIA were added in triplicates to sterile 96-well polypropylene plates and mixed with mid-log–phase BM110 strain BM110 in a dose-dependent manner. Importantly, the killing of GBS was washed once in HEPES buffer and incubated with 100 μl of sPLA2, final concentration 12.5 μg/ml, for 10 min at 37°C. The bacteria were washed three times in PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter. Radiolabeled sPLA2-IIA bound to JRS4 was then eluted by adding 1 ml of 1 M NaCl and incubating the tube at RT for 30 min. The tube was centrifuged, and the supernatant was diluted 100-fold (+10^5 CFU/ml) in HEPES buffer. After incubation at RT for 10 min, bacteria were washed twice with PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter. Radiolabeled sPLA2-IIA bound to JRS4 was then eluted by adding 1 ml of 1 M NaCl and incubating the tube at RT for 30 min. The bacteria were washed three times in PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter. D, Overnight cultures of JRS4 and BM110 were grown to mid-log phase (OD_{620} = 0.4). JRS4 was washed once in HEPES buffer and incubated with 100 μl of sPLA2, final concentration 12.5 μg/ml, for 10 min at 37°C. The bacteria were washed three times in 200 μl HEPES buffer. Recombinant sPLA2-IIA was then eluted from the bacteria by adding 100 μl of 1 M NaCl and incubating the tube at RT for 30 min. The tube was centrifuged, and the supernatant was diluted ×10, ×100, and ×500 in HEPES buffer, and a killing assay using GBS strain BM110 was performed. Aliquots of sPLA2-IIA were added in triplicates to sterile 96-well polypropylene plates and mixed with mid-log–phase BM110 diluted 100-fold (∼1 × 10^5 CFU/ml) in HEPES buffer. After incubation at 37°C for 30 min, the samples were diluted in PBS and plated onto blood agar plates to determine the bacterial viability by colony counting. The killing is presented as the percentage of CFU for GBS strain BM110 remaining in the wells after incubation with eluted sPLA2-IIA compared with the bacterial inoculum. The inhibitor LY311727 was used to confirm that the killing of BM110 is mediated by sPLA2-IIA.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Human sPLA2-IIA shows greater binding to WT *S. pyogenes* compared with the SrtA- mutant and retains its bactericidal activity. A, Mid-log–phase bacteria were washed in HEPES buffer and suspended to a 5% bacterial suspension (∼5 × 10^9 CFU/ml). Bacteria were serially diluted and added to radiolabeled sPLA2-IIA (125I)sPLA2-IIA; ∼10,000 cpm). After incubation at RT for 10 min, bacteria were washed twice with PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter. B, Mid-log–phase bacteria were washed in HEPES buffer and suspended to a 1% suspension (∼1 × 10^9 CFU/ml). Bacteria were mixed with various amounts of [125I]sPLA2-IIA (50,000, 10,000, and 2,000 cpm) and incubated at 37°C for 10 min. The bacteria were washed twice in PBSAT, boiled in sample buffer for 5 min, and subjected to SDS-PAGE, after which proteins were blotted onto a nitrocellulose membrane and analyzed by autoradiography. C, Mid-log–phase JRS4 was washed in HEPES buffer and diluted to a 1% bacterial suspension (∼1 × 10^9 CFU/ml). Bacteria were then added to radiolabeled sPLA2-IIA (∼35,000 cpm). After incubation at RT for 10 min, bacteria were washed twice with PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter. Radiolabeled sPLA2-IIA bound to JRS4 was then eluted by adding 1 ml of 1 M NaCl and incubating the tube at RT for 30 min. The bacteria were washed three times in PBSAT, and radioactivity associated with the bacterial pellet was measured with a gamma counter. D, Overnight cultures of JRS4 and BM110 were grown to mid-log phase (OD_{620} = 0.4). JRS4 was washed once in HEPES buffer and incubated with 100 μl of sPLA2, final concentration 12.5 μg/ml, for 10 min at 37°C. The bacteria were washed three times in 200 μl HEPES buffer. Recombinant sPLA2-IIA was then eluted from the bacteria by adding 100 μl of 1 M NaCl and incubating the tube at RT for 30 min. The tube was centrifuged, and the supernatant was diluted ×10, ×100, and ×500 in HEPES buffer, and a killing assay using GBS strain BM110 was performed. Aliquots of diluted sPLA2-IIA were added in triplicates to sterile 96-well polypropylene plates and mixed with mid-log–phase BM110 diluted 100-fold (∼1 × 10^5 CFU/ml) in HEPES buffer. After incubation at 37°C for 30 min, the samples were diluted in PBS and plated onto blood agar plates to determine the bacterial viability by colony counting. The killing is presented as the percentage of CFU for GBS strain BM110 remaining in the wells after incubation with eluted sPLA2-IIA compared with the bacterial inoculum. The inhibitor LY311727 was used to confirm that the killing of BM110 is mediated by sPLA2-IIA.

and IL-8 (42, 47, 48). To analyze whether sPLA2-IIA is inactivated when bound to JRS4, we analyzed the bactericidal activity of sPLA2-IIA against the GBS strain BM110 after elution from JRS4. As shown in Fig. 5D, sPLA2-IIA eluted from JRS4 retained its bactericidal properties, because it was capable of killing GBS strain BM110 in a dose-dependent manner. Importantly, the killing was specifically mediated by sPLA2-IIA, because it was completely inhibited by LY311727. Therefore, it is unlikely that the mechanism for protection against sPLA2-IIA in JRS4 is by proteolytic cleavage and inhibition of the bactericidal activity of sPLA2-IIA. This was further supported by the finding that the sPLA2-IIA eluted from JRS4 did not appear to differ in size compared with that eluted from the SrtA- mutant or the control sPLA2-IIA not preincubated with bacteria (Fig. 5B).

*SrtA does not protect S. pyogenes against positively charged AMPs*

As mentioned above, D-alanylation of LTA was shown to contribute to resistance to both sPLA2-IIA and positively charged AMPs. Because sPLA2-IIA is a highly positively charged molecule, it is possible that the SrtA-mediated resistance mechanism against sPLA2-IIA might also affect the sensitivity to AMPs, most of which are highly basic. To examine whether SrtA also contributes to the resistance to AMPs, we analyzed the difference in sensitivity between the WT JRS4 strain and the isogenic SrtA- mutant to the positively charged human cathelicidin LL-37 and Magainin II. As shown in Fig. 6, there was little or no difference in sensitivity to LL-37 or Magainin II between JRS4 and the SrtA- mutant, indicating that the SrtA-mediated resistance mechanism in *S. pyogenes* is specific for sPLA2-IIA.

*S. pyogenes SrtA is important for resistance against sPLA2-IIA in vivo*

To determine whether SrtA is important for *S. pyogenes* to be able to resist sPLA2-IIA in vivo, we used Tg mice overexpressing human sPLA2-IIA (Tg sPLA2-IIA). These mice were generated in the WT C57BL/6 background, in which there is a natural mutation in the *Pla2g2a* gene encoding endogenous mouse sPLA2-IIA (37). The Tg sPLA2-IIA mice contain high levels of human sPLA2-IIA activity in serum and various organs (Table I), whereas no sPLA2-
S. pyogenes against the bac tericidal activity of positively charged AMPs. Bacteria were grown to mid-log phase and directly diluted 1000-fold into HEPES buffer to give a bacterial concentration of \( \sim 1 \times 10^7 \text{CFU/mL} \). Serial dilutions of LL-37 (A) or Magainin II (B) in HEPES buffer (10 \( \mu \text{l} \)) were mixed with the bacterial suspension (10 \( \mu \text{l} \)). The samples were incubated at 37°C in 5% \( \text{CO}_2 \) for 2 h, plated onto blood agar plates, and incubated overnight to determine the bacterial viability by colony counting. The killing is presented as the percentage of CFU for each bacterial strain remaining in the wells after incubation with LL-37 or Magainin II compared with the inoculum.

Human sPLA2-IIA confers host protection against lethal S. pyogenes infection

To investigate the role of human sPLA2-IIA in the defense against lethal S. pyogenes infection, we infected C57BL/6 and Tg sPLA2-IIA mice i.p. with a 1:1 mixture of JRS4 and SrtA" bacteria. Compared with the WT strain JRS4, the SrtA" mutant was eliminated to a slightly higher degree in WT C57BL/6 mice, because almost 7-fold more of the WT JRS4 strain was found in spleen and liver after 18 h (Fig. 7). Strikingly, this difference was significantly more pronounced in the Tg sPLA2-IIA mice compared with the WT JRS4. Importantly, we demonstrated that SrtA contributes to the protection against sPLA2-IIA. The SrtA" mutant was significantly more effective in the Tg sPLA2-IIA mice, in which the WT strain JRS4 was not protected against sPLA2-IIA. We hypothesized that S. pyogenes use one or more LPXTG surface protein(s) that specifically bind and sequester sPLA2-IIA at the bacterial surface, thereby preventing this enzyme from reaching the cellular phospholipid membrane. Indeed, we demonstrated that WT S. pyogenes bacteria showed 5-fold higher binding to sPLA2-IIA compared with the SrtA" mutant, supporting this hypothesis. An alternative explanation for the greater association of sPLA2-IIA to JRS4 compared with the SrtA" mutant could be that sPLA2-IIA is internalized to a greater extent into the WT JRS4 bacteria. To exclude this possibility, we showed that >90% of the bound sPLA2-IIA dissociated from the WT bacteria when incubated in a high-salt buffer, providing strong evidence that most sPLA2-IIA is bound to the bacterial surface in JRS4. We also showed that sPLA2-IIA bound to WT S. pyogenes retains its bactericidal properties, providing further evidence that the major mechanism to resist sPLA2-IIA is to sequester this molecule at the bacterial surface.

To analyze the role of S. pyogenes SrtA in the resistance against sPLA2-IIA-mediated killing in vivo, we developed a mouse model of mixed infection measuring the CI, which gives the relative contribution of individual bacterial factors to virulence (41, 50, 51). In this model, we showed that the ratio of JRS4/SrtA" bacteria found in liver and spleen is significantly higher in the output from Tg sPLA2-IIA mice compared with C57BL/6 mice. This showed that SrtA provides a significantly greater selective advantage to S. pyogenes in mice expressing human sPLA2-IIA compared with WT mice lacking expression of sPLA2-IIA, most likely by protecting the bacteria against direct killing by sPLA2-IIA. Importantly, because there was no apparent difference in inflammatory status between Tg sPLA2-IIA and WT C57BL/6

<table>
<thead>
<tr>
<th>Tissue/Body Fluid</th>
<th>sPLA2-IIA Enzymatic Activity (cpm/ml/min; mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>Ear</td>
<td>14,494 ± 1,222</td>
</tr>
<tr>
<td>Spleen</td>
<td>11,047 ± 957</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>6,162 ± 525</td>
</tr>
<tr>
<td>Liver</td>
<td>10,140 ± 1,154</td>
</tr>
<tr>
<td>Lungs</td>
<td>11,137 ± 1,525</td>
</tr>
<tr>
<td>Serum</td>
<td>9,567 ± 595</td>
</tr>
</tbody>
</table>

Table 1. Measurement of sPLA2-IIA enzymatic activity in sPLA2-IIA Tg mice (n = 8)
mice during the experiment, we can rule out that the difference in CI is due to different levels of inflammation between the two types of mice. Of note, the concentration of human sPLA2-IIA in serum from Tg sPLA2-IIA mice (∼2500 μg/l) is comparable to levels of sPLA2-IIA measured in serum from human patients with severe sepsis (880 μg/l) or typhoid fever (1440 μg/l) (14, 52, 53), indicating that the level of human sPLA2-IIA in Tg mice is physiologically relevant.

Other investigators have successfully used SrtA– mutants as the basis for analysis of the role of individual LPXTG proteins in interactions between Gram-positive pathogens and their infected host. For example, in a mouse model of oral infection, Lalioui et al. (54) showed that an SrtA– mutant strain of GBS was strongly impaired in ability to colonize the intestine, a location that is a natural habitat for GBS in humans. In a subsequent study, it was demonstrated that a single LPXTG protein, HvgA, is responsible for the ability of GBS to colonize the gut (55). In another study, Cheng et al. (56) used an SrtA– mutant of S. aureus in analyses demonstrating that LPXTG proteins were required for this pathogen to cause kidney abscess formation in the mouse model, which led to the identification of the LPXTG protein SdrD as the main virulence factor in this model. Thus, the use of SrtA– mutants as the basis for studies on the interaction between Gram-positive pathogens and their host has revealed important functions for individual LPXTG proteins. Among the LPXTG proteins in S. pyogenes are several potential virulence factors that could be responsible for the resistance to sPLA2-IIA. Although we cannot rule out that more than one LPXTG protein contributes to the resistance, we hypothesize that a single LPXTG protein is responsible for the protection against sPLA2-IIA. Indeed, the ligand-binding properties of LPXTG proteins in interactions with host proteins very often are highly specific, with well-defined binding domains in the bacterial proteins (26, 40, 42, 57, 58).

Although S. pyogenes is a clinically important pathogen, we still know little about the in vivo role of different antibacterial molecules in the defense against S. pyogenes. In a study by Nizet et al. (59), it was shown that mice deficient in the cathelicidin CRAMP, the mouse equivalent of LL-37, are more sensitive to skin infections caused by S. pyogenes. A recent study by Shannon et al. (60) showed that the histidine-rich glycoprotein, an abundant plasma protein, protects mice against S. pyogenes infection by the s.c. route, a property that may be due, at least in part, to its antibacterial activity. In this study, we showed that Tg mice over-expressing human sPLA2-IIA are significantly protected against lethal S. pyogenes infection compared with WT C57BL/6 mice. This result showed that human sPLA2-IIA contributes to the host defense against serious experimental S. pyogenes infection and provides a novel example of an antibacterial molecule that may be involved in the protection against S. pyogenes infections in humans.

FIGURE 7. SrtA is important for the ability of S. pyogenes to resist sPLA2-IIA in vivo. WT C57BL/6 or Tg sPLA2-IIA mice were infected i.p. with a 1:1 mixture of mid-log–phase JRS4 WT and SrtA– strains. This mixture was defined as the input. Each mouse received a total of ∼5 × 10⁸ CFU. Surviving C57BL/6 mice (n = 11) and Tg sPLA2-IIA mice (n = 10) were sacrificed at 18 h, after which the spleens and livers were collected. The organs were homogenized, serially diluted in PBS and plated on blood agar. The bacteria in these samples, defined as the output, were analyzed for presence of the two strains in the input. For each organ, a total of 100–200 colonies was picked and grown in short patches on THY agar containing 100 μg/ml spectinomycin. Only the SrtA– mutant strain grows on these plates because it contains a spectinomycin resistance gene not present in the WT strain. The CI was defined as the ratio between the JRS4 and SrtA– strains in the output divided by the same ratio in the input. ***p ≤ 0.001.

FIGURE 8. Human sPLA2-IIA protects mice against lethal infection with S. pyogenes. The WT strain JRS4 was grown to mid-log phase and diluted in ice-cold THY. Groups of six WT C57BL/6 and Tg sPLA2-IIA mice were infected i.p. with ∼8 × 10⁸ CFU of WT JRS4 bacteria, representing a 90% lethal dose in the WT C57BL/6 mice. Mice were monitored every 4 h, and deaths were recorded daily. The difference in lethality between C57BL/6 and Tg sPLA2-IIA mice was calculated with the two-tailed Fischer exact test (p = 0.03).
Acknowledgments
We thank Dr. Jane Scott and Dr. Gunnar Lindahl for providing bacterial strains used in the study.

Disclosures
The authors have no financial conflicts of interest.

References


