Serum amyloid A exhibits pH dependent antibacterial action and contributes to host defense against *Staphylococcus aureus* cutaneous infection

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Running title: Serum amyloid A and antibacterial

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Abstract
Serum amyloid A (SAA), one of the major highly conserved acute phase proteins in most mammals, is predominantly produced by hepatocytes and also by a variety of cells in extra-hepatic tissues. It is well known that the expression of SAA is sharply increased in bacterial infections. However, the exact physiological function of SAA during bacterial infection remains unclear. Herein, we showed that SAA expressed significantly increased in abscesses of Staphylococcus aureus (S. aureus) cutaneous infected mice, which exert direct antibacterial effects by binding to the bacterial cell surface and disrupting the cell membrane at acidic condition. Mechanically, SAA disrupts anionic liposomes by spontaneously forming small vesicles or micelles under acidic conditions. Especially, the N-terminal region of SAA is necessary for membrane disruption and bactericidal activity. Furthermore, we found that mice deficient of SAA1/2 was more susceptible to infection by S. aureus. In addition, the expression of SAA in infected skin was regulated by IL-6. Taken together, these findings support a key role of the SAA in host defense and may provide a novel therapeutic strategy for cutaneous bacterial infection.
**Introduction**

Serum amyloid A (SAA) is a major acute phase protein and highly conserved from sea cucumber to human. SAA increases rapidly in serum during the acute-phase response and is thought to be involved in innate immunity and lipid homeostasis during inflammation. Three isoforms of acute-phase SAA have been reported in mice, called SAA1, SAA2, and SAA3. However, the third gene, SAA3, is a pseudogene in human. The acute-phase SAAs in human and mouse are synthesized largely by hepatocytes upon inflammatory condition (1,2). SAAs are also widely expressed in normal extra-hepatic tissues (3), and significantly increased in inflammatory tissue (4-6). Furthermore, the plasma extravasation may enable delivery of hepatocyte-derived SAA to the infection site. On-site SAA may play a role in protecting the blood and other organs from disseminating microbes (7,8). SAA has long been used as a clinical biomarker for acute infections, and also has been reported to be expressed in keratinocytes and sebocytes and upregulated in some inflammatory cutaneous diseases (3,9,10), such as psoriasis, papulopustular acne and papulopustular rosacea. Although SAA has similar expression pattern with typical cutaneous antimicrobial proteins and peptides (AMPs) (11,12), its role in skin surface defense remains unclear.

The acidic condition (pH 4-6) is an important character of skin surface. Because of the bactercidal efficacy of many skin AMPs depends on the acidic pH of the skin, acidity of the stratum corneum and sweat is important for antimicrobial activity (13) Local acidosis also occurs during inflammation due to massive infiltration of neutrophils and macrophages at the site of inflammation, which subsequently activates respiratory burst, and hypoxia at sites of inflammation leading to local accumulation of lactic acid and a significant decrease in pH (14,15).

Many members of the SAA protein family are amphipathic and positively charged, which are typical characteristics of AMPs. In some species, such as goldfish and common carp, SAAs have a highly alkaline isoelectric point and present antimicrobial activity *in vitro* (16,17). *In vitro* antibacterial assays at neutral pH do not reveal that human or mouse SAAs have bactericidal properties (6). However, overexpression of mouse SAA1 and SAA2 (mSAA1 and mSAA2) in cultured epithelial cell lines reduces co-cultured *Escherichia coli* (*E. coli*) via an undefined mechanism (18). These works suggest that the local microenvironment of inflammatory foci may affect the bactercidal properties of SAA.

In this study, we demonstrated that SAA exhibits direct antibacterial activity at low pH condition through targeted disrupting bacterial membrane structures which are negatively charged phospholipids. Intriguingly, SAA rapidly self-assembles
with anionic phospholipids forming small vesicles or micelles under a mildly acidic pH condition. Moreover, SAA was strongly induced in *S. aureus* infected skin and reduced bacteria burden in vivo.

**Results**

*SAA is strongly induced in *S. aureus* infected skin and presents antimicrobial properties by disrupting bacterial membranes*

We first evaluated the expressions of SAA1/2/3 in an *S. aureus* cutaneous infected mouse model and found that mRNA levels of SAAs were significantly increased both in the skin and liver of the infected mice (Fig. 1A and Fig. S1). The expression of SAA was also detected by immunohistochemical staining. We found that all of three mice SAAs were abundantly expressed in cutaneous abscesses (Fig. 1B). To investigate whether SAA has a direct bactericidal effect, we tested the activity of SAA against *E. coli* and *S. aureus*, which are representatives for Gram-negative and Gram-positive bacterial strains, respectively. We found that bactericidal activity of mSAA1 was pH dependent (Fig. 1C), with the higher activity at acidic pH. The survival rates of both *E. coli* and *S. aureus* declined by 90% after incubation with 2.5 μM mSAA1 at pH 5.5 (Fig. 1D). We also tested the antibacterial activity of hSAA1, hSAA2, mSAA2 and mSAA3, the bactericidal activity of all these proteins increased significantly at pH 5.5 (Fig. 1E, F). To further validate the interaction between SAA and bacteria, we used serum from AgNO₃-injected mice (endogenous mSAA) and the purified recombinant mSAA1 (exogenous) to incubate with *E. coli* and *S. aureus* at different pH conditions. The binding abilities of endogenous mSAA and the purified recombinant mSAA1 to *E. coli* and *S. aureus* at acidic pH (pH 5.5) were strongly enhanced (Fig. 2A and B). Since the induced expression of recombinant SAA in bacteria causes bacterial cell lysis, suggesting the destructive effect of SAA on bacterial cell membranes (19). To investigate the direct interaction between SAA and bacteria, membrane integrity of mSAA1-treated bacterial cells was observed by using transmission electron microscopy (TEM). Drastic morphological changes in the bacterial cells were observed after incubating mSAA1 at low pH, and cell membrane damage and component leakage in both *S. aureus* and *E. coli* cells and cytoplasm condensation in *E. coli* cells were evident (Fig. 2C-E).

*SAA induces anionic liposome leakage by forming micelles under acidic conditions.*

The phospholipid binding specificity of SAA was examined by measuring releasing entrapped terbium (Tb³⁺) from liposomes of defined composition (20). Negatively charged phospholipids, such as phosphatidylglycerol (PG) and cardiolipin,
are abundantly present in bacterial cell membranes (21,22). To further understand the interaction between SAA and bacterial cell membranes, unilamellar liposomes were used as an in vitro model. No phosphatidylcholine (PC)-only liposomes leakage was observed with 5 μM mSAA1 and hSAA2 under neutral or acidic conditions, whereas 100% leakage in liposomes containing 20% cardiolipin (cardiolipin liposomes) and weaker leakage in liposomes containing 20% PG (PG liposomes) were observed with 5 μM mSAA1 and hSAA2 under neutral or acidic conditions. Moreover, mSAA1, mSAA2, hSAA1 and hSAA2 also induced negative-charged liposomes disruption under acidic condition. In order to investigate the interaction between SAA and cardiolipin, we performed the Bio-Layer Interferometry (BLI) to explore the binding affinity of proteins and liposomes. The biotinylated SAA protein was immobilized on a Streptavidin (SA) sensor, which was then flown through with the different liposomes. There was no observable binding to PC liposomes, but both mSAA1 and hSAA2 bound to cardiolipin liposomes were prominent (Fig. 3E, F). As the cardiolipin could not dissociate from SAA proteins, we speculated that SAA formed a stable complex with cardiolipin at acidic pH. Consistent with the liposome leakage assays, no interactions between SAA and PC or cardiolipin at neutral pH were observed.

To determine the exact interaction between SAA and liposomes, we performed TEM on negatively stained liposomes treated with mSAA1 at different pH conditions, we observed that SAA induced PG or cardiolipin liposomes collapse at acidic pH. Furthermore, the SAAs rapidly formed small vesicles or micelles with large scale disruption of the cardiolipin liposomes and PG liposomes under acidic conditions, and no significant morphological changes were observed in PG or cardiolipin liposomes at neutral pH and in PC liposome under both pH conditions (Fig. 4 and Fig. S2B). This indicated that SAA binds anionic phospholipids to form small vesicles or micelles and induce liposome leakage under an acidic pH condition.

The N-terminal region of SAA is necessary for liposome disruption and bactericidal activity.

Since the structure of hSAA1 and mSAA3 had been determined. The known monomer structure of hSAA1 and mSAA3 is very similar, which contains a cone with four-α-helix and a tail (23,24). By analyzing the surface hydrophobicity of SAA, a hypothetical model of SAA binding to HDL suggested that helix1 and helix3 of SAA form a hydrophobic surface that binds to HDL (25). Because of the N-terminal of SAA is important for HDL-binding, we speculated that the N-terminal region of SAA was also essential for its membrane
disruption activity. A mutation of 20 amino acids truncation of mSAA1 (ΔN20-mSAA1) was constructed. We found that the ΔN20-mSAA1 mutation did not induce PG and cardiolipin liposomes leakage (Fig. 5A-D). The negative stain EM also showed that the truncation mutation did not induce cardiolipin liposomes disruption (Fig. 5E). Furthermore, the interaction with cardiolipin liposomes was detected by BLI. It was also found that under acidic conditions, ΔN20-mSAA1 could not bind to cardiolipin liposomes (Fig. 5F). Consequently, the bactericidal activity of truncation mutation was also significantly impaired (Fig. 5G).

**Deletion of Saa1/2 in mice impaired the clearance of Staphylococcus aureus during cutaneous infections.**

To further explore the role of SAA in host defense we generated Saa1/2 double knockout mice (SAA1/2 DKO) and mice to test the function for endogenous SAAs in host defense against cutaneous bacterial infections. We verified that SAA1/2 was absent in the skin of SAA1/2 DKO mice (Fig. 6A) and showed that *S. aureus* infection led to higher numbers of cutaneous bacteria and larger abscesses area in the absence of SAA1/2 (Fig. 6B and C). Since IL-6 is critical for the acute-phase response to bacterial infection. Hepatic production of the SAA was markedly diminished during bacterial infection in *Il6*Δ/Δ mice (26). Real-time PCR analysis of IL-6 also showed that mRNA levels for IL-6 were markedly increased in the infected skin (Fig. 6D). We assumed that the production of SAA in skin induced by *S. aureus* was IL-6 dependent. To test this hypothesis, we infected the wild-type (WT) and *Il6*Δ/Δ mice with *S. aureus*. Compare to the WT mice, mRNA levels of SAAs were significantly decreased in skin of the infected *Il6*Δ/Δ mice (Fig. 6E). Taken together, we demonstrated that SAA has antibacterial activity both in vitro and in vivo and regulated by IL-6.

**Discussion**

SAA has long been recognized as a major acute phase response protein in inflammatory diseases, but its molecular actions of innate immunity during bacterial infection remain largely unclear. In the current study, we demonstrated that SAA was abundantly expressed in cutaneous abscesses when infected with *S. aureus*. SAA not only binds to phospholipids of bacterial membranes through its hydrophobic N terminal region, but exerts direct bactericidal by disrupting bacterial cell membrane under acidic conditions. Furthermore, we demonstrated that the antimicrobial effects of SAA against skin
bacterial infections in vivo, which may provide a novel therapeutic strategy for cutaneous bacterial infection.

The SAA protein family is highly conserved, and the crystal structure reveals that SAA contains a four-α-helix in which helix1 and helix3 form a hydrophobic surface that binds hydrophobic ligands, such as phospholipids cholesterol and retinol (24). Some conserved charged amino acid residues interact with polysaccharides and cell receptors (25), suggesting that SAA exerts multiple functions at the inflammatory site, where the concentration of SAA rises sharply during the acute response. SAA, during the acute phase, is primarily associated with high density lipoprotein (HDL) (27). Under mild acidic conditions, heparan sulfate binds to SAA and causes differential dissociation of SAA from the HDL particle, but has no effect on the HDL-associated ApoA-I (28). A recent study also showed that SAA solubilized phospholipid bilayers to form lipoproteins that provided substrates for sPLA2 and effectively removed free fatty acids under acidic conditions (29). Inflammatory foci are characterized by low pH levels reaching as low as pH 5 (30), indicating that the microenvironment at inflammatory foci may influence the biochemical properties and physiological function of SAA. Increased SAA expression was observed in biopsies from patients with Crohn’s disease and other inflammatory diseases (4,18), and such increased SAA was co-localized with bacteria in animal model (6,31), which has been speculated to play a role in bacterial sensing and killing mechanisms to protect tissues of host.

It has been reported that SAA is able to bind to Gram-negative but not Gram-positive bacteria under neutral conditions and acts as an opsonin (32). In this study, we found that SAAs bind both Gram-positive and Gram-negative bacteria, disrupt the bacterial cell membrane, which in turn cause bacterial cell death under the mild acidic pH of the local inflammatory site. AMPs have three different strategies to insert and disrupt the cell membrane, by forming a barrel stave or toroidal pore to make the membrane permeable, by forming a ‘carpet model’ which causes the accumulation of peptides or proteins on the bilayer surface, leading to disruption of the bilayer in a detergent-like manner, eventually leading to the formation of micelles (33). We demonstrated that SAA was electrostatically attracted to the anionic phospholipid head groups at the surface of the membrane and disrupted cell membrane in a carpet-like manner by its hydrophobic surface under an acidic pH condition. These results consisted with that a group of AMPs show activity against microbes that present low pH optima (30,34,35).

In this study, we also found the N-terminal region of mSAA1 is necessary for phospholipids binding and bactericidal activity. Consist with our results, Patel et al. found that deletion of the first 11 amino acid
residues at the N-terminus of recombinant human SAA diminishes its capacity to bind to HDL and decreases amyloid fibril formation (36). And another group had reported that the N-terminal region (residues 1–27), of human SAA1 is important for lipid interaction and sufficient for phospholipid binding (37). Previous studies have reported that oligomerized SAA formed a channel on the planar membrane and was associated with toxic amyloid oligomers (19,33,38). However, we did not observe channels formation on PG liposomes incubated with SAAs. In contrast, SAAs bound PG liposomes and cardiolipin liposomes, but not PC liposomes, and spontaneously formed small vesicles or micelles under acidic conditions, like a typical apolipoprotein. A reasonable explanation is that SAAs bind to anionic phospholipids with a higher affinity than a zwitterionic phospholipid under acidic conditions. Like other lipoproteins, the amphipathic SAA helix bundle forms a stable HDL-like nanoparticle with the zwitterionic phospholipid; however, this nanoparticle requires a high protein/phospholipid ratio and longer incubation time under neutral conditions (39), a low protein/lipid ratio and short time does not make morphological changes in vesicles (40). In addition, other studies have reported that some HDL components can be involved in the resistance to microbial infections, in which apoL-1 can selectively bind anionic phospholipids under acidic conditions to disrupt cell membranes and kill trypanosomes (41), while apoA-1 is also have a higher affinity to anionic phospholipids and inhibit bacterial growth (22). The results of this study indicate that SAA has similar biochemical and biological functions to these apolipoproteins and provides new evidence for the important role of HDL in host defense.

S. aureus, a major cause of skin and soft-tissue infections in human, which causes both local and systemic diseases (42,43). In this study, we found that SAA was strongly induced in infected skin of an S. aureus cutaneous infected mouse model. We also found that both SAA1/2 DKO and mice have higher bacterial loads in cutaneous abscesses after subcutaneous S. aureus infection. Furthermore, the production of SAA in infected skin was IL-6 dependent. Consisted with our results, many studies showed that IL-6 was critical for acute phase response and acute phase proteins (APPs) production (26,44). Previously studies showed SAA played a role in host defense of Salmonella typhimurium and Klebsiella pneumoniae infection (24,45). Our data demonstrated that SAA also participated in host defense of S. aureus cutaneous infection in vivo. SAA has also been reported to regulate immune cells, such as promoting the secretion of cytokines such as IL-17, IL-22 (46,47), and recruiting neutrophils and monocytes (48), whether the immunomodulatory function of SAA is also involved in the resistance against S. aureus infection remains to be
confirmed by further studies.

Although no case report has identified a genetic human SAA deficiency, evidence suggests that many of patients with liver dysfunction have defective acute-phase responses to bacterial infection, contributing to an increased susceptibility to bacterial infection (49). SAA plays a pivotal role in regulating inflammatory responses, suggesting that SAA may not only be the major source of acute-phase proteins and a clinical marker, but a functional immune response player that provides a novel therapeutic strategy for inflammation during the innate immune response to infection. While as the large-scale clinical trial to validate its direct innate immune effects is needed.

In summary (Fig. 7), we demonstrated that SAA not only bound to anionic phospholipids of bacterial cell membrane and self-assembled to form micelles but elicited direct bactericidal effects through a hydrophobic N-terminal region as well. Our study also confirmed that SAA is abundantly expressed in S. aureus infection sites and protects from S. aureus cutaneous infection in vivo.

Materials and Methods

Animals

All mice were housed and bred in the specific pathogen-free facility at China Agricultural University. 8–12 weeks old male mice were used for all experiments. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of China Agricultural University (approval number: SKLAB-2017-01-05). Saa1 and Saa2 double knockout mice (C57BL/6 background) were generated by Nanjing biomedical research institute of Nanjing University. SAA1/2 double knockout mice were generated a 15kb chromosomal deletion at Saa1 & Saa2 locus in the mouse genome using CRISPR/Cas9 technology. IL-6 deficient (Il6−/−) mice (C57BL/6 background) were obtained from Dr. Zhinan Yin, Jinan University, Guangzhou.

Microorganisms

Bacterial isolates E. coli BL21 and S. aureus ATCC 25923 were obtained from China General Microbiological Culture Collection Center, CGMCC Beijing.

Antibodies and reagents

All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). The polyclonal antibody for mSAA1/2 was purchased from R&D Systems (Minneapolis, MN, USA). The polyclonal antibodies for human SAA1/2 (hSAA1/2) and mouse SAA3 (mSAA3) was purchased from ABclonal (Wuhan, China). Terbium chloride (TbCl3) and DPA were purchased from Sigma-Aldrich (St. Louis, MO, USA), and
all other chemicals were from Sigma-Aldrich.

Mouse serum sample preparation

Ten-week-old male C57BL/6 mice were intraperitoneally injected with AgNO₃ (0.5 mL, 0.01 g/mL). After 24 h, serum was obtained by centrifugation (50).

Gene cloning, protein expression and purification

The human and mouse SAA cDNA encoding 103 amino acid residues were cloned into pET21a with an N-terminal 6× His tag followed by tobacco etch virus (TEV) protease cleavage site and a C-terminal stop codon. Recombinant pET-SAA plasmids were transformed into E. coli strain BL21 (DE3) pLysS (Tiangen, China). The cells were grown at 37°C in LB medium supplemented with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol until OD600 reached about 0.6. Protein expression was induced with 0.5 mM isopropyl-β-D-galactoside (IPTG) and incubated at 25°C, after 30 min; 100 μg/ml rifampicin was added to inhibit E. coli RNA polymerase. After an additional 2.5 h incubation, the cells were harvested and resuspended in lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, pH 8.0 for mSAA1, mSAA2, mSAA3, hSAA1 and hSAA2), then a 0.5% final concentration of n-dodecyl-β-d-maltoside (DDM) was added to the lysis buffer and incubated for 3 h at 4°C after sonication. The lysate was clarified by centrifugation at 42,000 × g for 30 min at 4°C. The supernatant was loaded onto a Ni Sepharose TM (GE Healthcare, USA) column pre-equilibrated with lysis buffer containing 0.05% DDM. The column was washed at least 40 times with 25 mM imidazole in lysis buffer to completely remove non-specific contaminants and detergent (21), and the proteins were eluted in lysis buffer containing 300 mM imidazole. The eluted product was pooled, and buffer exchanged into 20 Mm Tris-HCl, pH 8, 200 mM NaCl, and then digested with TEV protease at 18°C for 12 h. Undigested protein was removed by passing the protein through a Ni affinity matrix. Whereas digested protein was collected only the flow through. The eluate was concentrated and further purified with a Resource Q column (GE Healthcare, USA) and HiLoad 16/600 Superdex™200pg column (GE Healthcare, USA) in 100 mM NaCl, 20 mM Tris pH 8, 1 mM TCEP, and 5% glycerol. Finally, the purified protein was > 95% pure according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE).

Mutagenesis

Deletion mutant was generated using standard PCR methods. Protein expression and purification were done as for wild-type SAA.
Viable count analysis

Bacteria were grown overnight in Luria-Bertani (LB) medium. The microbes were washed twice with 10 mM sodium phosphate, 150 mM NaCl buffer, pH 7.4, and diluted in the same buffer at a pH range of 5.2–7.4. Then, about $10^7$ CFU microbes were incubated at 37°C for 2 h at the indicated concentrations with SAA. To quantify bactericidal activity, serial dilutions of the incubated mixtures were plated on LB (for bacteria), followed by an incubation at 37°C overnight, and the number of colony-forming units was determined. Then, 100% survival was defined as total survival of bacteria in the same buffer and under the same conditions as in the absence of proteins.

Binding assay

*E. coli* and *S. aureus* ($10^8$ CFU) were incubated with 5 μM mSAA1 in 500 μL 10 mM sodium phosphate, 150 mM NaCl, pH 5.5 or pH 7.4 for 1 h at 37°C, centrifuged, and the pellets were washed three times in the same buffer. The pellet and the supernatant were resuspended in SDS sample buffer, electrophoresed (15% SDS-PAGE), and then transferred to a PVDF membrane with a transfer buffer (25 mM Tris, 192 mM glycine, and 20% [v/v] methanol). The membranes were blocked with 5% dried milk in TBS-T (20 mM Tris, 500 mM NaCl, 0.05% Tween 20) and incubates with mSAA1 antibody for 1 h at room temperature. Then secondary antibody (rabbit anti goat HRP; 1:5,000; Santa Cruz, US), was diluted into hybridization buffer and incubated at room temperature for 1 h. The blot was then washed five times for 5 min with wash buffer and HRP was detected with ECL reagent (GE Healthcare, US).

Liposome preparation

The method for liposome preparation of this study was modified from Ding et al (20). In brief, different components of phospholipids were separately dissolved in chloroform. The lipid of the specified fraction (0.5 μmol) was mixed in a glass vial. The solvent was evaporated under a stream of nitrogen, and the dried lipid film was hydrated at room temperature and mixed in 500 μL of buffer 1 (20 mM HEPES [pH 7.4] or 20 mM MES [pH 5.5] and 150 mM NaCl). The Tb$^{3+}$-packaged liposomes were hydrated in 500 μL of buffer 2 (20 mM HEPES [pH 7.4] or 20 mM MES [pH 5.5]), 100 mM NaCl, 50 mM sodium citrate and 15 mM TbCl3. Liposomes were generated by repeated pushes of hydrated lipids through a 100 nm polycarbonate filter (Whatman, UK) 35 times using a Mini-Extruder apparatus (Avanti Polar Lipids, USA). After the push process, the liposomes were added to a centrifugal filter tube (Amicon Ultra-4, 100K MWCO; Millipore, USA) and the Tb$^{3+}$ ions outside the liposome were removed by repeated washings 5 times with Tb$^{3+}$-free buffer 2. The liposomes were replaced with buffer 1 for use.
Lipid leakage assay

Lipid leakage assay was implemented for this study was modified from Ding et al (20). In brief, a 30 μL aliquot of Tb³⁺-packaged liposomes was mixed with 60 μL of DPA-containing buffer 1 and 10 μL of the specified concentration of SAA recombinant protein was added before assay. The final concentration of phospholipid was 300 μM and the concentration of DPA was 15 μM. The Tb³⁺/DPA chelate was examined using excitation and emission wavelengths of 270 nm and 490 nm, respectively, in a full-wavelength fluorescence microplate reader. The emission fluorescence before the addition of the SAA protein was regarded as Ft0. A 10 L aliquot of the protein was added to the designated and the emission fluorescence was continuously recorded as Ft at 30 s intervals. After 20 min, 10 μL of 1% Triton X-100 was added to achieve complete release of Tb³⁺, and the average of the first three fluorescence readings was defined as Ft100, if the fluorescence value of the protein treatment group was greater than after adding 1% Triton X-100. The fluorescence value takes three maximum fluorescence values of Ft100. The percentage of liposome leakage at each time point is defined as:

\[ \text{Leakage (t) (\%) = \frac{(F_t - F_{t0}) \times 100}{(F_{t100} - F_{t0})} } \]

Transmission electron microscopy

Mid-logarithmic phase E. coli and S. aureus cells (OD600 at 0.4) were incubated with 10 μM mSAA1 at pH 5.5 for 2 h. After treatment, the bacterial pellets were prefixed with 2.5% glutaraldehyde at pH 7.4 for 2 h at 4°C and post-fixed in 1% osmium tetroxide buffered at pH 7.4 for 2 h at 4°C. The samples were dehydrated in acetone (50, 70, 90, 95, and 100%). The cells were immersed in EPON resin, and ultrathin sections were examined under a JEOL JEM-1230 (Jeol Ltd., Japan). The leakage area of bacterial cell component was quantified by Image J (NIH).

Negative staining electron microscopy of the SAA-lipid micelles

SAA proteins (5 μM) were incubated with the indicated liposomes (500 μM lipids) at room temperature for 30 min. Aliquots of the mixture (5 μL) were transferred to a glow-discharged carbon-coated copper grid for EM and negatively stained with 2% uranyl acetate. Samples were imaged on a JEM-1400 electron microscope (Jeol Ltd., Japan) at 120 kV.

Biolayer interferometry

The Biolayer interferometry analysis was performed using Octet Red 96 System (Fremont, USA) with SA chips at room temperature 25 °C. All assays were performed in 10 mM sodium phosphate 150
mM NaCl pH 5.5 or 7.4. SAA proteins were biotinylated, then flowed through with 100 μM PC liposome or cardiolipin liposome in different buffers.

**Immunohistochemistry**

This method was performed as previously described (51). Briefly, four-micron tissue sections were deparaffinized in xylene and rehydrated through a series of decreasing ethanol concentrations. The slides were pretreated with hydrogen peroxide (3%) for 10 min to remove endogenous peroxidase, followed by antigen retrieval in a microwave for 15 min in 10 mM citrate buffer (pH 6.0). The primary antibodies were applied, followed by washing and incubation with the biotinylated secondary antibody for 30 min at room temperature. The slides were counterstained with hematoxylin and dehydrated in alcohol and xylene before mounting.

**RNA extraction and real-time RT-PCR**

Total RNA and quantitative real-time PCR (qPCR) were performed as described previously (51). Data were analyzed with LightCycler® 480 software, Version 1.5 (Roche). Relative quantification of gene expression was performed using the standard curves and normalized to the value for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each sample. Primers for Saa1/2, Saa3, Il6 and Gapdh were used in supplemental Table 1.

**Cutaneous infection in vivo**

Mouse model of cutaneous infection was modified from Li et al (52) and Zhang et al (43). In brief, the backs of age-matched adults were shaved, and hair was removed by using chemical depilation. In the same day *S. aureus* was grown in LB medium for overnight. Next day, 1 mL of overnight culture of *S. aureus* was re-inoculated into 30 mL fresh LB and grew to logarithmic phase (OD600 = 0.7–0.8). Then the bacterium centrifuged, and the pellets were washed and resuspended in sterile PBS. $3 \times 10^6$ CFU live *S. aureus* was subcutaneously injected into mouse back skin (n=5 per each group). Mice were euthanized after four days. In some experiments, skin around the abscess was collected and homogenized in PBS to determine the number of surviving *S. aureus*. In other experiments, RNA from normal or infected skin was collected either for real-time RT-PCR or stored in 4% paraformaldehyde for immunostaining. Samples were randomized during data collection. Investigators are not blinded to the group allocation during data acquisition.

**Statistics**

Data were analyzed for statistical significance with SPSS 12.0.1. All
numerical data were presented as mean ± SEM (standard error of mean). A $P$ value less than 0.05 was considered significant.

All graphs were generated with GraphPad Prism 6.0 (GraphPad Software Inc.)

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Figure legends

Fig. 1. The expression of SAA in the S. aureus cutaneous infection model and the antibacterial activity of SAA to E. coli and S. aureus in a pH dependent manner.

A. SAA mRNA expression in skin and liver after mouse back skin infected with $3 \times 10^6$ CFU S. aureus (n=5) in day 3. B. Immunohistochemistry analysis of SAA in mouse skin after S. aureus infection in day 3 (scale bar, 200 μm). C. Bactericidal activity of mSAA1 at different pH values was measured against E. coli and S. aureus using a microdilution susceptibility assay (n=3). 100% survival was defined as total survival of bacteria in the same buffer and under the same conditions as in the absence of proteins. D. Percentage of CFU remaining after exposure to purified mSAA1. S. aureus and E. coli were grown to mid-log phase and incubated with purified proteins. After incubation for 2 h at pH 5.5 at 37°C, the viability of bacteria was quantified by dilution plating (n=3). E-F. Bactericidal activity of different SAA proteins, the viability of bacteria was quantified by dilution plating (n=3). All data are representative of three independent experiments. Significant differences versus control group were presented by asterisks (*), * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 2. SAA binds bacteria and disrupts its cell membrane at low pH.

A. Binding of mSAA1 to E. coli or S. aureus was incubated with 5 μM recombinant mSAA1 in 10 mM sodium phosphate, 150 mM NaCl buffer pH 5.5 or pH 7.4. After centrifugation, the supernatant (S) and the pellet (P) were analyzed by SDS–PAGE and Coomassie blue staining. B. Binding of mSAA1 to E. coli or S. aureus was incubated with 10% AgNO3-injected mice serum in 10 mM sodium phosphate, 150 mM NaCl buffer pH 5.5 or pH 7.4. After centrifugation, SAA in the pellet was detected by immunoblotting. C. Transmission electron microscopy micrographs of E. coli and S. aureus cells were incubated with 10 μM mSAA1 or buffer. (scale bar,500 nm). D-E. Cell component leakage areas of S. aureus (D) and E. coli (E) cells (n=30) were incubated with 10 μM mSAA1 or buffer. Significant differences versus control group were presented by asterisks (*), ***$P < 0.001$.

Fig. 3. SAA binds to negatively charged lipids and induced liposome leakage.

A-B. SAA disrupted terbium (Tb$^{3+}$) loaded unilamellar liposomes containing the negatively charged lipid phosphatidyl glycerol (PG) or cardiolipin (Card), but not liposomes composed of the zwitterionic lipid phosphatidylcholine (PC). Liposomes were treated with 5 μM mSAA1 (A) or hSAA2 (B). after incubated with SAA proteins. Detergent was added after 20
min. C-D.PG (C) and Cardiolipin (D) liposome-leakage-inducing activity of mSAA1, mSAA2, hSAA1 and hSAA2. E. Octet binding response of PC liposomes and cardiolipin liposomes to mSAA1 or hSAA2 at pH 5.5. F. Octet binding response of PC liposomes and cardiolipin liposomes to mSAA1 or hSAA2 at pH 7.4. The proteins were immobilized onto a SA sensor followed by dipping these biosensors into the liposome solution and the protein liposome interaction was represented as binding curves showing the association. All data are representative of three independent experiments.

**Fig. 4. Membrane disruption and micelles nanoparticles formation activity of SAA.**

PG liposomes (left), cardiolipin liposomes (middle) and PC liposomes (right) after 1 h of incubation with mSAA1 at pH 7.4 (Bottom) or pH 5.5 (Top) at 37 °C. Protein: lipid molar ratio was 1:100. Shown were representative negative-stain electron microscopy micrographs of the liposomes (scale bar,100 nm). Black arrows indicate small vesicles or micelles. All data are representative of three independent experiments.

**Fig. 5. The structure analysis of SAA shows N-terminal of SAA is necessary for liposome disruption and bactericidal activity.**

A-D. Effects of ΔN20-mSAA1 mutation on mSAA1 liposome-leakage-inducing activities. A. Purified mSAA1 and ΔN20-mSAA1 (10 μM) proteins were incubated with cardiolipin liposomes. B. Means ± SEM from three independent replicates of the experiment shown in A. C. mSAA1 and ΔN20-mSAA1 (1 μM) were incubated with PG liposomes at pH 5.5. D. Means ± SEM from three independent replicates of the experiment shown in C. E. Negative-stain electron microscopy micrographs of cardiolipin liposomes incubated with mSAA1 and ΔN20-mSAA1 mutation at pH 5.5 (scale bar,100 nm). Protein: lipid molar ratio was 1:100. F. Octet binding response of cardiolipin liposomes to ΔN20-mSAA1 at pH 5.5. G. Effects of ΔN20-mSAA1 mutation on mSAA1 antibacterial activity (n=3). All data are representative of three independent experiments. Significant differences versus control group were presented by asterisks (*), **P < 0.01.

**Fig. 6. Deletion of Saa1/2 in mice impaired the clearance of Staphylococcus aureus in the skin.**

A. Wild type and SAA1/2DKO littermates were analyzed at 8 weeks of age by Q-PCR for Saa1/2 gene expression in S. aureus infected skin (n=5). B. Bacterial counts from S.
*aureus*-infected wild-type and SAA1/2 DKO mice cutaneous abscesses. C. Abscesses areas of *S. aureus*-infected wild-type and SAA1/2 DKO mice. Mice were subcutaneously infected for 4 day (n=4-5). D. IL-6 mRNA levels in the infected skin were determined by Q-PCR and are expressed relative to the levels in uninfected WT mice. (n=5). E. SAA mRNA expression in skin of wild-type and *Il6*−/− mice infected with *S. aureus* (n=3-5). Significant differences versus control group were presented by asterisks (*), *P* < 0.05; **P** < 0.01.

**Fig. 7. Graphic summary.**

The bacterial killing mechanism of SAA under acidic condition. SAA bounds to anionic phospholipid of bacterial membrane and disrupts cell membrane by self assembling to form micelles through the hydrophobic N-terminal region.
**Fig. 1**

**A** Liver

![Graph showing relative Saa expression in liver with PBS and S. aureus treatments.]

**B** Skin

![Images showing tissue sections at pH 7.4 and pH 5.5 with PBS and S. aureus treatments.]

**C** Skin

![Graph showing relative Saa expression in skin with PBS and S. aureus treatments.]

**D** S. aureus and E. coli survival at pH 7.4 and pH 5.5 with mSAA1 treatment levels.

**E** S. aureus

![Graph showing survival of S. aureus with mSAA2, mSAA3, hSAA1, and hSAA2 at pH 7.4 and pH 5.5.]

**F** E. coli

![Graph showing survival of E. coli with mSAA2, mSAA3, hSAA1, and hSAA2 at pH 7.4 and pH 5.5.]

*Significant differences indicated by asterisks: * p < 0.05, ** p < 0.01, *** p < 0.001.
Fig. 2

A

S. aureus  E. coli

kDa

|     | S | P |     | S | P |
|-----|---|---|-----|---|---|
| 70  |   |   | 35  |   |   |
| 55  |   |   | 25  |   |   |
| 40  |   |   | 15  |   |   |
| 30  |   |   | 10  |   |   |

pH

|     | S | P |     | S | P |
|-----|---|---|-----|---|---|
| 7.4 |   |   | 5.5 |   |   |
| 7.4 |   |   | 5.5 |   |   |

B

S. aureus  E. coli

pH

|     | S | P |     | S | P |
|-----|---|---|-----|---|---|
| 7.4 |   |   | 5.5 |   |   |
| 7.4 |   |   | 5.5 |   |   |

mSAA1

kDa

|     |     |     |     |     |
|-----|-----|-----|-----|
| 15  | 10  | 10  | 10  |

Serum

kDa

|     |     |     |     |
|-----|-----|-----|
| 15  | 10  | 10  |

C

S. aureus

E. coli

Buffer  mSAA1

Leakage area of cell component (%)

S. aureus

buffer  mSAA1

***

E. coli

buffer  mSAA1

***
Fig. 3

A  mSAA1

B  hSAA2

C  PG liposome: 80% PC + 20% PG

D  Cardiolipin liposome: 80% PC + 20% cardiolipin

E  PC liposome

F  PC liposome

Cardiolipin liposome
Fig. 4

pH 5.5

pH 7.4

PG liposome  Card liposome  PC liposome
Fig. 5

A

B

C

D

E

F

G
Fig. 6

A. Relative SAA1/2 mRNA levels

B. CFU per abscess

C. Areas (mm²)

D. Relative IL-6 mRNA levels

E. Relative Saa1/2 expression

F. Relative Saa3 expression
Fig. 7
Serum amyloid A exhibits pH dependent antibacterial action and contributes to host defense against Staphylococcus aureus cutaneous infection
Han Zheng, Haifeng Li, Jingyuan Zhang, Hanlu Fan, Lina Jia, Wenqiang Ma, Shuoqian Ma, Shenghong Wang, Hua You, Zhinan Yin and Xiangdong Li

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