Targeting Surface Protein SasX by Active and Passive Vaccination To Reduce *Staphylococcus aureus* Colonization and Infection

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SasX is a recently described surface protein of *Staphylococcus aureus* that is linked to the epidemic success of hospital-associated methicillin-resistant clones, in particular in Asia. It enhances nasal colonization and virulence in skin and lung infection models. Here, we evaluated the potential of SasX as a vaccine component in passive and active immunization efforts using mouse infection models. We found that SasX induced a specific immune response predominantly based on IgG1 antibodies. Active immunization with recombinant SasX or passive immunization with rabbit polyclonal anti-SasX IgG significantly decreased the size of lesions caused by *S. aureus* in a skin infection model. Furthermore, active immunization reduced acute lung injury in a lung infection model. Moreover, active or passive immunization significantly reduced *S. aureus* colonization in a nasal colonization model. Finally, anti-SasX IgG enhanced the susceptibility of *S. aureus* to killing by human neutrophils. We conclude that SasX is a potential target for therapeutics or vaccines designed to moderate colonization and infection by *sasX*-positive epidemic strains of *S. aureus*.

*S*taphylococcus aureus is a major global source of morbidity and mortality (1), causing more than 11,000 deaths per year in the United States alone (2). It is particularly notorious as a dangerous hospital-associated pathogen. Treatment of *S. aureus* infections is severely complicated by antibiotic resistance (3). In particular, resistance to methicillin in methicillin-resistant *S. aureus* (MRSA), which in many countries occurs in more than half of infectious *S. aureus* isolates, is a major health care concern. In addition, many *S. aureus* strains are resistant to a wide variety of other antibiotics, leaving only very limited options for treatment. In an era that has seen a broad withdrawal of pharmaceutical companies from the much-needed development of novel antibiotics, researchers in companies and academia are again beginning to attempt vaccine development against *S. aureus*. A working vaccine against *S. aureus* infections is not available, and multiple reasons have been discussed for why an anti-*S. aureus* vaccine is difficult to find (4, 5). These include first and foremost the large arsenal of immune evasion factors of *S. aureus*, which target both acquired and innate immune defenses (6). However, there is promising evidence indicating that passive and active vaccination against *S. aureus* should in principle be possible, notwithstanding the fact that we still do not completely understand the mechanisms the immune system uses for protective immunity against *S. aureus* (4).

Infective *S. aureus* and MRSA isolates are very diverse regarding geographical origin and time of isolation. Over the years, specific predominant MRSA clones arose and were thereafter replaced by others, in a scenario of epidemic waves (7). Furthermore, infections in different geographic areas are characterized by a divergent and often endemic composition of prevalent *S. aureus* and MRSA lineages. This situation requires an adaptation of vaccine targets to specific, predominant infectious clones.

The sequence type 239 (ST239) lineage of MRSA isolates is the predominant lineage causing hospital-associated infections in Asia (8). Furthermore, it has caused outbreaks in other geographical locations (9). We previously showed that many ST239 isolates harbor a lysogenic prophage expressing a surface protein, SasX, which is associated with disease severity in skin and lung infections (10). It also facilitates nasal colonization, from which infection can originate. Notably, the *sasX* gene has been spreading at a considerable rate among ST239 and other MRSA lineages in Chinese hospitals and is considered an important factor contributing to the pathogenic success of the ST239 lineage (10). In the present study, we evaluated active and passive immunization strategies using the SasX protein to reduce infectivity and colonization by ST239 and other *sasX*-containing MRSA.

MATERIALS AND METHODS

Ethics statement. All animal work was approved by the ethics committee of Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. Human neutrophils were isolated from the heparinized venous blood of healthy individuals with a standard method (11) in accordance with a protocol approved by the ethics committee of Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. All individuals gave informed consent prior to donating blood.

Bacteria and growth conditions. Bacteria were identified as staphylococci by classic microbiological methods: Gram's staining and catalase and coagulase activity on rabbit plasma. *S. aureus* strains were

further categorized by biochemical characterization using the Api-Staph test (bioMérieux, Lyon, France). The MRSA clinical isolate HS770 (ST239, containing the *sasX* gene) was recovered from the sputum of an inpatient with pneumonia at Shanghai Hospital, China, and determined to belong to ST239 by multilocus sequence typing (MLST) ([10]). Isolate HS770 was grown in tryptic soy broth (TSB; Oxoid) and used in all animal work. *Escherichia coli* BL21 was grown in Luria-Bertani broth (LB; Oxoid). When necessary, media were supplemented with ampicillin (100 μg/ml for *E. coli*) or chloramphenicol (10 μg/ml) for *S. aureus*.

Construction and purification of recombinant SasX protein. The *sasX* gene was cloned, overexpressed, and purified as a glutathione *S*-transferase (GST) fusion protein as described elsewhere ([10]). To obtain pure, recombinant SasX (rSasX) for use in immunization experiments, the GST part was removed by thrombin digestion ([Fig. 1A] and [B]).

Production of rabbit antisera and purification of anti-SasX IgG. Purified rSasX protein was used as an immunogen for the production of rabbit polyclonal antisera (provided by Youke Biotech Company, Shanghai, China). Anti-SasX IgG was purified using the protein A affinity column Hitrap rProteinA FF (GE Healthcare) using an AKTA purifier (GE Healthcare) according to the manufacturer's specifications.

Active immunization. Female BALB/c mice were used for active immunization. All mice were 5 to 6 weeks of age. The mice were randomly allocated to 3 treatment groups as follows: (i) rSasX plus Freund's adjuvant (Sigma), (ii) inactivated *S. aureus* HS770 plus Freund's adjuvant, and (iii) phosphate-buffered saline (PBS) (control group). Purified rSasX protein was dissolved in PBS and emulsified in Freund's adjuvant; the emulsions (100 μl each) contained 50 μg of protein. The inactivated *S. aureus* vaccine was prepared as follows. MRSA HS770 was cultured overnight at 37°C in TSB, harvested by centrifugation, resuspended in sterilized PBS, and adjusted to 1×10^6 CFU/ml. The bacteria were then inactivated at 37°C for 24 h in 0.2% (wt/vol) formaldehyde and emulsified at a 1:1 ratio in complete Freund's adjuvant (CFA). The mice were immunized by intramuscular injection of 50 μg of purified rSasX or inactivated *S. aureus* vaccine in CFA on day 0, followed by a boost with the same antigens in incomplete Freund's adjuvant (IFA) at 12-day intervals (days 0, 12, and 24). Blood samples were collected on days 7, 19, 31, and 38, and the sera were harvested and stored at −80°C. The control group was injected with PBS by following the same protocol.

Detection of specific IgG by ELISA. Enzyme-linked immunosorbent assays (ELISAs) were used to determine the titers of IgG against rSasX. Ninety-six-well ELISA plates (JET BIOFIL; JET Biotech Company, Guangzhou, China) were coated with 100 μl of rSasX at a concentration of 1 μg/ml overnight at 4°C in carbonate/bicarbonate buffer (pH 9.6). Then the plates were blocked using 100 μl carbonate/bicarbonate buffer (pH 9.6) containing fetal calf serum (FCS) (10% [wt/vol]) overnight at 4°C. The serum samples were added at serial dilutions and incubated for 1 h at 37°C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (1:2,000) were then added, and the mixture was incubated for 1 h at 37°C. A total of 100 μl chromogenic substrate solution (42 mM tetramethylbenzidine and 0.01% hydrogen peroxide) was added to the plates. Plates were incubated at 37°C for 10 min. Then the enzymatic reaction was stopped by the addition of 50 μl of 2 N hydrofluoric acid. Three washes using PBS with 0.05% Tween 20 were performed between each step. The optical density (OD) at 450 nm/630 nm was read on a microplate reader (BioTek Instruments, Winooski, VT). The method used to determine the titers of antibodies against the SasX in inactivated *S. aureus* was the same as that described above for rSasX, except that the coated antigen was inactivated *S. aureus*.

Detection of the subtypes of antibodies. ELISAs were performed as in the method described for the detection of specific IgG, except the secondary antibody was mouse anti-IgG1–HRP (1:500), mouse anti-IgG2a–HRP (1:500), mouse anti-IgG2b–HRP (1:500), mouse anti-IgG3–HRP (1:500), or mouse anti-IgM–HRP (1:500) (Southern Biotech).

Mouse cytokine detection. Cytokine concentrations in the blood or supernatant of the homogenized tissues of mice were detected using a
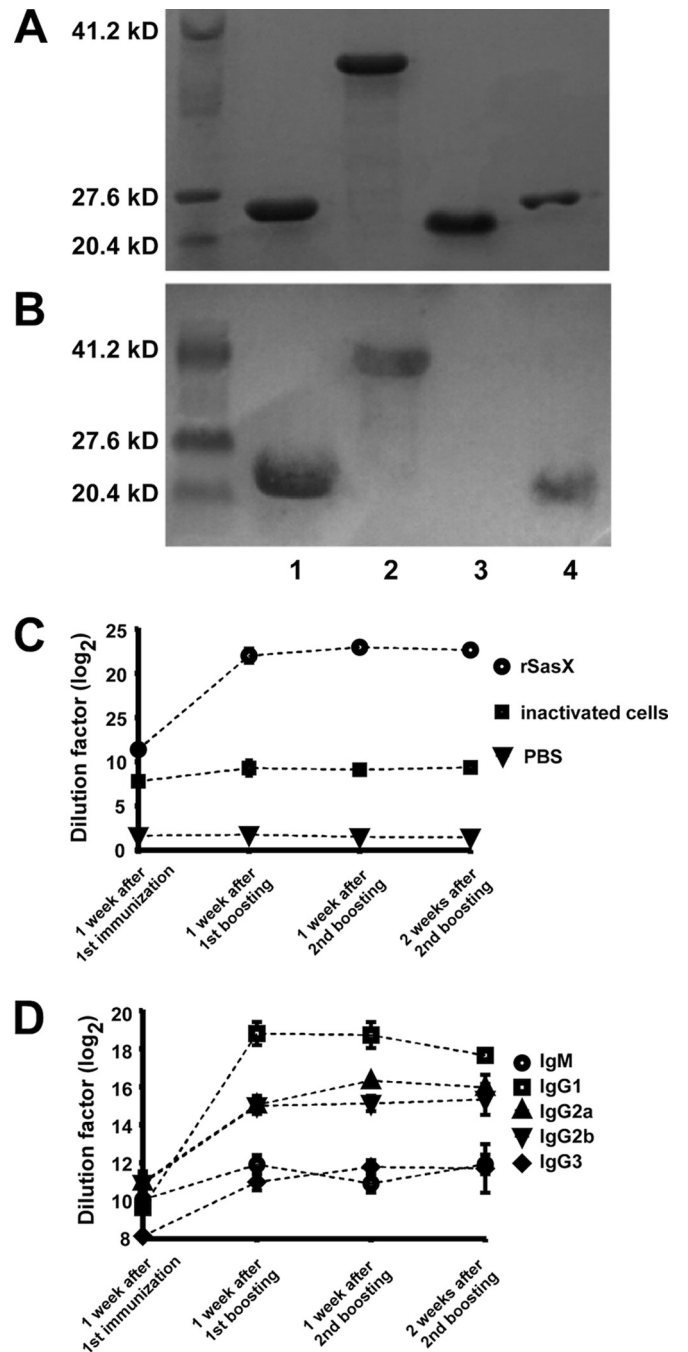

FIG 1 Recombinant SasX can induce specific immune responses. (A and B) Production and purification of rSasX. Lane 1, purified GST protein; 2, purified GST-SasX fusion protein; 3, purified rSasX protein after thrombin digestion, which was used for immunization and vaccination; 4, purified GST-tagged protein after thrombin digestion. The molecular mass marker is at the left. SDS-PAGE with Coomassie blue staining is shown in panel A, and a Western blot with anti-GST antibodies is shown in panel B. (C) IgG titers against rSasX in serum determined by ELISA. (D) Serum concentrations of antibody subtypes determined by ELISA. The mice were immunized three times at 12-day intervals (days 0, 12, and 24), and sera were obtained from the mice 1 week after the first immunization, 1 week after the first boosting, 1 week after the second boosting, and 2 weeks after the second boosting. Inactivated *S. aureus* cells and rSasX were emulsified in Freund's adjuvant. The control group was injected with only PBS.

Bio-Plex Pro assay kit (Bio-Rad Laboratories). A total of 50 µl of antibody-conjugated beads was added to the assay plate. The samples were diluted 1:4, 50 µl of diluted samples, standards, the blank, and controls was added to the plate, and the plate was incubated in the dark at room temperature (RT) with shaking at 300 rpm for 30 min and then washed 3 times with 100 µl wash buffer. A total of 25 µl biotin-ylated antibody was added to the plate, which was incubated in the dark at RT with shaking at 300 rpm for 30 min and then washed 3 times with 100 µl wash buffer. A total of 50 µl streptavidin-phycoerythrin (PE) was added to the plate. The plate was again incubated in the dark at RT with shaking at 300 rpm for 10 min and then washed 3 times with 100 µl wash buffer. Finally, the plate was read using a Bio-Plex protein array reader. Bio-Plex Manager 6.0 software was used for Bio-Plex Pro assay data acquisition and analysis.

Skin abscess model in mice after active immunization. The skin abscess model was performed 1 week after the first boosting during the active immunization procedure. Before the experiments, the mice were shaved around the site of injection. The HS770 isolate was grown to mid-exponential phase, washed once with sterile PBS, and then resuspended in PBS at 1×10^9 CFU/100 µl. Mice ($n = 15$ per group) were anesthetized with isoflurane and inoculated with 100 µl PBS containing 10^9 live *S. aureus* cells in the right flank by subcutaneous injection. Ten mice were chosen randomly out of the 15 mice per group once subcutaneous injection of *S. aureus* was finished to measure abscess formation and were examined with a caliper at 24-h intervals for a total of 10 days. We applied length (L) and width (W) values to calculate the area of abscesses with the formula $L \times W$. On the fourth day after infection, the remaining five mice per group were killed, the abscess regions were excised, and part of the excised tissue material was fixed in 10% formalin (Sigma). Paraffin embedding, hematoxylin and eosin (H&E) staining, and toluidine blue (TB) staining were performed as described previously (10, 12–14). Abscess material samples of 0.05 g were homogenized in 0.5 ml PBS for the measurement of cytokines. All animals were euthanized after completion of the entire procedure.

Skin abscess model after passive immunization. Female BALB/c mice were used for passive immunization. All mice were 5 to 6 weeks of age. The mice were randomly allocated to 2 treatment groups ($n = 15$ per group), as follows: (i) rabbit polyclonal anti-SasX IgG and (ii) rabbit preimmune serum. The mice received 5 mg total IgG purified from either the rabbit preimmune serum or the polyclonal anti-SasX IgG in 500 µl PBS via intraperitoneal injection. Twenty-four hours later, the mice received another 5 mg total IgG via intraperitoneal injection. Twenty-four hours later, the mice in each group were anesthetized with isoflurane and inoculated with 100 µl PBS containing 10^9 live HS770 bacterial cells in the left flank by subcutaneous injection. Subsequent steps were the same as for the active immunization procedure.

Lung infection model in mice after active immunization. The lung infection model was performed 1 week after the first boosting during the active immunization procedure. Isolate HS770 was grown to mid-exponential growth phase, washed, and resuspended in sterile PBS at 5×10^7 CFU/µl. Mice ($n = 15$ per group) were anesthetized with isoflurane. The inoculum, which contained 10^9 CFU/20 µl, was pipetted into the nares of the anesthetized mice without touching the nose. Mice were euthanized at 24 h ($n = 4$), 48 h ($n = 8$), or 72 h ($n = 3$) after infection. The lungs were excised and washed with saline. The right lungs were homogenized in 0.5 ml of TSB, the homogenized lung tissue was plated in triplicate on tryptic soy agar (TSA) containing 5% sheep blood, and the supernatant of the homogenized lung tissue was used for cytokine detection. The left lung was fixed in 4% formalin (Sigma). Paraffin embedding and H&E staining were performed as previously described (10, 12).

Nasal colonization model in mice after active immunization. The nasal colonization model was performed 1 week after the first boosting during the active immunization procedure. Isolate HS770 was grown to mid-exponential growth phase, washed, and resuspended in sterile PBS at 10^6 CFU/µl. Mice ($n = 24$ per group) were anesthetized with isoflurane.

The inoculum, which contained 10^7 CFU in 10 µl of PBS, was pipetted slowly into the nares of the anesthetized mice without touching the pipette tip to the nose. Three days and 7 days after inoculation, 12 mice per group were chosen randomly, euthanized, and evaluated for nasal carriage of *S. aureus*. The nasal region was wiped externally with 70% ethanol, and the nasal tissue was homogenized in 0.5 ml of TSB. The total number of *S. aureus* CFU per nose was assessed by plating 100 µl of diluted nasal suspensions on TSB agar containing penicillin (100 µg/ml).

Nasal colonization model in mice after passive immunization. The passive immunization procedure was performed as described above for the skin abscess model. For the preventive approach, 24 h after immunization, the mice in each group were anesthetized and received *S. aureus* bacteria as described above for the active immunization procedure in the nasal colonization model. On day 7 after inoculation, all mice were euthanized and evaluated for nasal carriage of *S. aureus* as described above. For the treatment experiment, procedures were the same except that mice first were given *S. aureus* in the nose and received 10 mg of preimmune serum or the polyclonal anti-SasX IgG serum via intraperitoneal injection 24 h afterwards.

Opsonophagocytic killing. Isolated human neutrophils were adjusted to 5×10^6/ml in RPMI 1640. The complement source (rabbit serum; Sigma) was adsorbed three times with HS770 bacteria at 4°C for 30 min in order to remove *S. aureus*-specific antibodies. After adsorption, the complement solution was centrifuged and filter sterilized. HS770 bacteria were grown to mid-exponential phase, washed once with sterile PBS, and resuspended in RPMI 1640 at 5×10^7 CFU/ml. The opsonophagocytic killing assay was performed by mixing 100 µl (each) of the neutrophil suspension, HS770 cells, dilutions of rabbit polyclonal anti-SasX IgG or rabbit preimmune serum, and the complement source. The reaction mixture was incubated at 30°C for 90 min. The samples were taken at 0 min, 30 min, 60 min, and 90 min and plated on TSA containing 5% sheep blood for detection of surviving *S. aureus*. Percent survival was determined relative to control reactions, to which no neutrophils were added. The lysis of neutrophils after phagocytosis was measured using a lactate dehydrogenase (LDH) cytotoxicity detection kit according to the manufacturer's protocol (Roche) as described elsewhere (15). Samples were diluted 1:20 for the assays.

Statistics. Statistical analysis was performed using Graph-Pad Prism, version 6.02, with unpaired t tests. Error bars show the standard error of the mean (SEM).

RESULTS

Vaccination with rSasX induces specific immune responses. To test whether rSasX induces specific immune responses in mice, IgG titers were measured after three rounds of vaccination (Fig. 1C). Compared to inactivated *S. aureus* immunization with the same adjuvant, rSasX induced a much stronger antibody response. In addition, rSasX stimulated a more rapid increase in antibody levels than inactivated *S. aureus*. Furthermore, we measured the serum levels of antibody subtypes directed against rSasX following the administration of rSasX emulsified in Freund's adjuvant (Fig. 1D). The subtype of antibodies induced by rSasX was predominantly IgG1. The titers of IgG1 induced by rSasX peaked at 1 week after the first boosting. These results show that rSasX is strongly immunogenic in mice, causing a rapid and substantial production of specific antibodies.

Opsonic activity of rabbit polyclonal anti-SasX IgG. For passive immunization efforts, we developed a polyclonal anti-SasX antiserum in rabbits and tested it first for its potential to stimulate killing after opsonophagocytosis. Opsonophagocytic killing by neutrophils is the primary mechanism by which *S. aureus* is cleared by the host's immune system (16). Opsonophagocytosis assays with human neutrophils can be used to assess the function
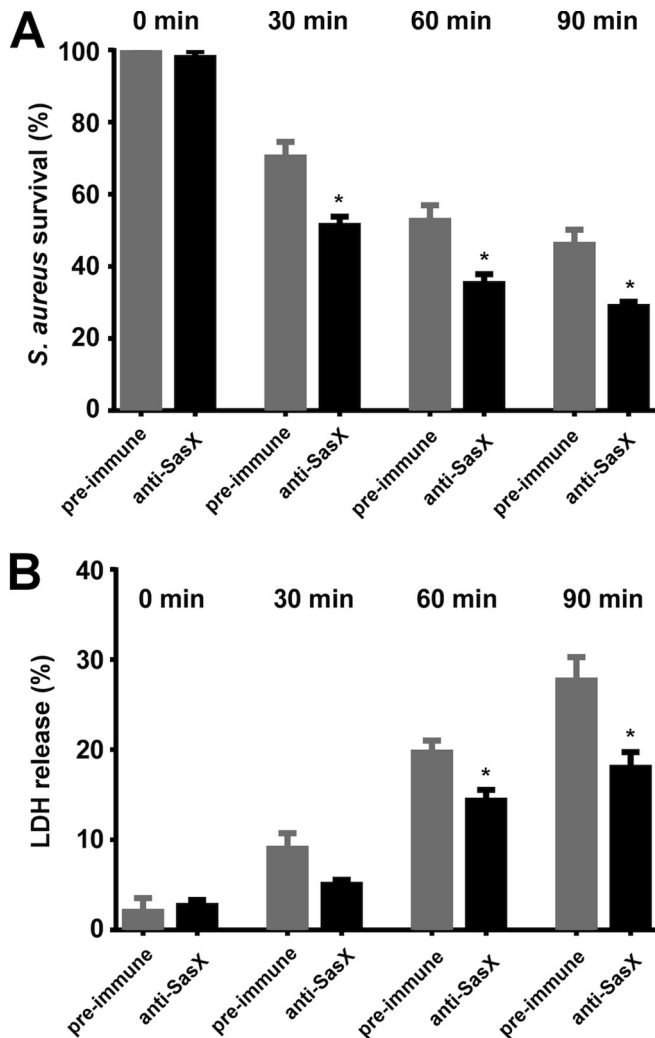
FIG 2 Rabbit polyclonal anti-SasX IgG enhances the susceptibility of MRSA ST239 toward elimination by human neutrophils. (A) Survival of *S. aureus* (isolate HS770) after *in vitro* opsonophagocytosis by human neutrophils. Bacteria were incubated for 90 min with neutrophils, the complement source, and dilutions of rabbit polyclonal anti-SasX IgG or rabbit preimmune serum. The number of surviving bacteria was determined by dilution plating. *, $P < 0.05$. (B) *In vitro* neutrophil lysis after opsonophagocytosis of *S. aureus* cells. Neutrophil lysis was determined by release of lactate dehydrogenase (LDH). *, $P < 0.05$.

of antistaphylococcal antibodies, because an *in vitro* opsonic reaction is initiated when bacterial cells, functional antibodies, and an exogenous complement source are mixed together. Measurement of *S. aureus* survival after opsonophagocytosis *in vitro* showed that a significantly lower number of bacteria survived upon the addition of purified rabbit polyclonal anti-SasX IgG than in rabbit preimmune serum-treated cultures (Fig. 2A). We also found a significant reduction in neutrophil lysis as measured by release of lactate dehydrogenase (LDH) upon the addition of rabbit polyclonal anti-SasX IgG, which can be interpreted as a result of a lower number of bacteria being present to lyse neutrophils by mechanisms such as toxin-mediated lysis (17) (Fig. 2B). These findings demonstrate that anti-SasX antibodies promote elimination of *sasX*-positive MRSA ST239 by human neutrophils.

Active or passive immunization with rSasX reduces skin infection in mice. We first analyzed whether active immunization with rSasX reduces skin infection due to MRSA ST239 using a mouse abscess model. Skin infections (wounds and abscesses) were the second most frequent type of infection caused by *sasX*-positive *S. aureus* in our previous study (10). Skin lesions of mice infected with MRSA isolate HS770 (ST239) were significantly smaller after active immunization with rSasX plus Freund's adjuvant than lesions of mice in PBS control-immunized groups (Fig. 3A and B). Furthermore, we measured the infiltration of neutrophils and mast cells, as they have been implicated in a variety of inflammatory conditions and play an important role in host defense against staphylococcal infection (14, 18). In the group of mice immunized with rSasX, the degree of skin tissue destruction and the degree of neutrophil and mast cell infiltration were strongly reduced compared to those in PBS control mice (Fig. 3C). It has been reported that mast cells modulate the influx of neutrophils and monocytes, and thus clearance of bacterial infection, through release of chemoattractants, such as tumor necrosis factor alpha (TNF-α) (13, 18). Concentrations of TNF-α and monocyte chemoattractant protein 1 (MCP-1) in the supernatant of homogenized abscess tissue were significantly lower in the rSasX-immunized group than in the PBS control group (Fig. 3D). These results show that active immunization with rSasX against *sasX*-positive MRSA ST239 results in reduced symptoms of skin disease, including dermonecrosis and inflammatory reactions.

We then also evaluated whether passive immunization with polyclonal anti-SasX IgG antibodies obtained from rabbits could reduce skin infection due to MRSA ST239. After 2 and 7 days of skin infection, mice that had received anti-SasX antibodies had significantly smaller lesions than mice that had received preimmune serum as a control (Fig. 3E and F). Furthermore, the treated mice showed reduced neutrophil infiltration and skin tissue destruction compared to those of the mice treated with preimmune serum (Fig. 3G). These results showed that passive immunization with anti-SasX antibodies can reduce skin infection due to *sasX*-positive MRSA ST239 and indicate that the protection achieved with active immunization was antibody mediated.

Active immunization with rSasX reduces lung infection in mice. In addition to infections of the skin, *S. aureus* is most commonly involved in respiratory infections (1). In the specific case of infections with *sasX*-positive *S. aureus*, respiratory infections were the predominant type of infections caused (10). Therefore, we determined whether active immunization against rSasX would moderate the severity of acute lung injury. Lung wet weight/body weight ratios (Fig. 4A), macroscopic and histological examination (Fig. 4B), bacterial counts (Fig. 4C), and the degree of inflammation in the lung as assessed by TNF-α and MCP-1 concentrations (Fig. 4D) showed that active immunization with rSasX reduces acute lung injury and inflammation during lung infection with *sasX*-positive MRSA ST239.

Active and passive immunization with rabbit polyclonal anti-SasX IgG reduces nasal colonization in mice. Nares are the predominant location of *S. aureus* colonization in the human body (19). Our previous study demonstrated that the SasX surface protein promotes nasal colonization (10). Therefore, we tested whether active immunization with rSasX or passive immunization with rabbit polyclonal anti-SasX IgG can reduce nasal colonization in a preventive or treatment fashion. The determination of
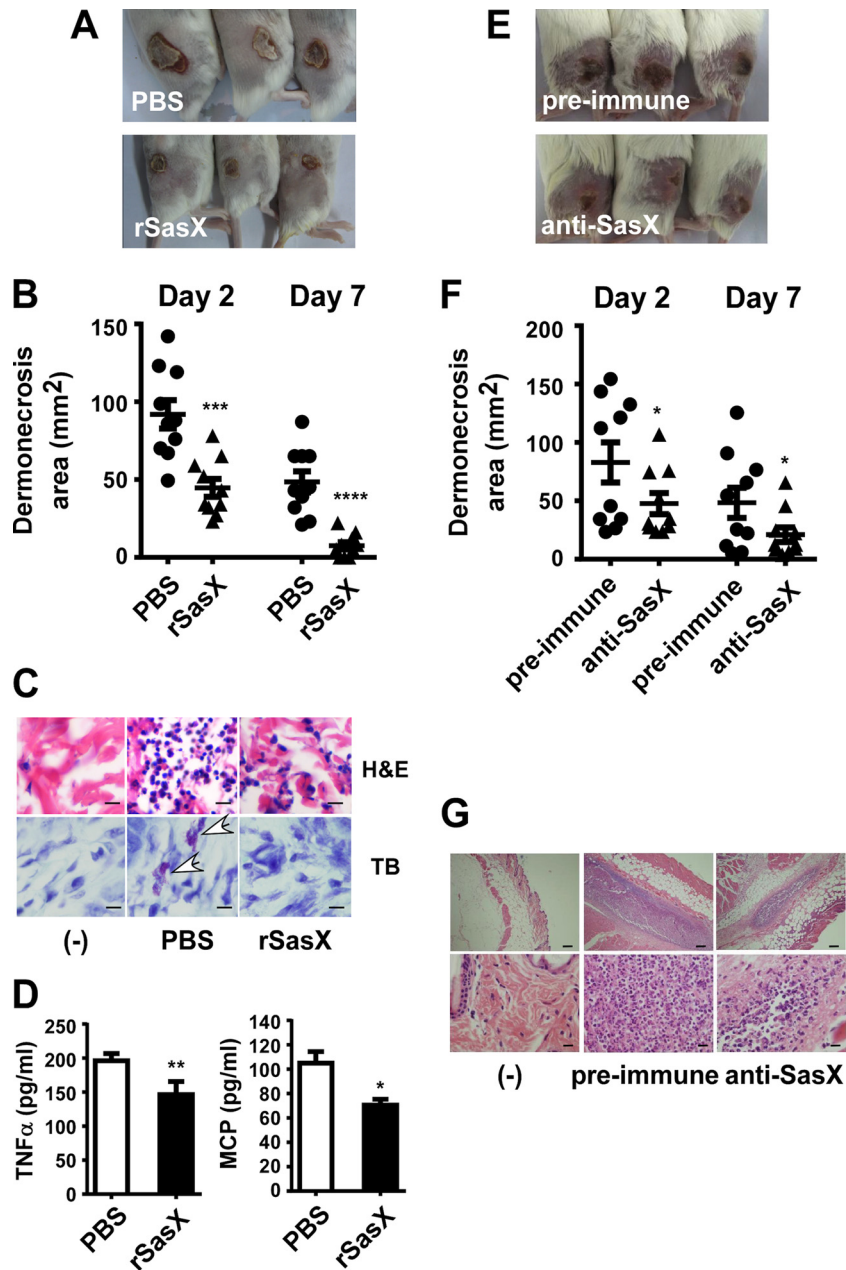
FIG 3 Active and passive immunization with SasX reduces dermonecrosis caused by MRSA ST239. To measure the outcome of active immunization, mice were challenged with 10^9 live *S. aureus* CFU 1 week after the first boosting with rSasX (or the PBS control), and dermonecrotic lesions were measured with a caliper at 24-h intervals for a total of 10 days. (A) Representative skin lesions on day 4; (B) dermonecrosis areas on days 2 and 7 after infection. ***, $P < 0.001$; ****, $P < 0.0001$. (C) H&E and TB staining of abscess skin tissues harvested on day 4. (−), control without bacterial challenge. Top row (H&E staining), neutrophils appear purple; bottom row (TB staining), mast cells appear purple (white arrows). Scale bars, 20 μm. (D) Cytokine concentrations in abscess tissue. The concentrations of the TNF-α and MCP-1 cytokines were measured by ELISA in abscess samples on day 4 after infection. *, $P < 0.05$; **, $P < 0.01$. To measure the outcome of passive immunization, mice received a total of 10 mg IgG of either rabbit preimmune serum or polyclonal anti-SasX IgG via intraperitoneal injection in 24-h intervals. Twenty-four hours later, the mice were inoculated with 100 μl PBS containing 10^9 live MRSA HS770 bacteria in the left flank by subcutaneous injection, and dermonecrotic lesions were measured with a caliper at 24-h intervals for a total of 10 days. (E) Representative skin lesions on day 4; (F) dermonecrosis areas on days 2 and 7 after infection. *, $P < 0.05$. (G) H&E staining of abscess skin tissues harvested on day 4. Scale bars, 20 μm (bottom row) and 200 μm (top row); (−), control without bacterial challenge.

bacterial counts after 7 days of inoculation of the noses of mice showed that rabbit polyclonal anti-SasX IgG had a significant preventive effect regarding subsequent colonization, compared with PBS and rabbit preimmune serum (Fig. 5A). Notably, anti-SasX IgG could also reduce colonization in a treatment fashion, i.e.,

once colonization was established. These results show that nasal colonization of *sasX*-positive MRSA ST239 can be suppressed using anti-SasX antibodies. Similarly, using an active immunization approach, vaccination with rSasX reduced subsequent colonization by *sasX*-positive MRSA ST239 (Fig. 5B).
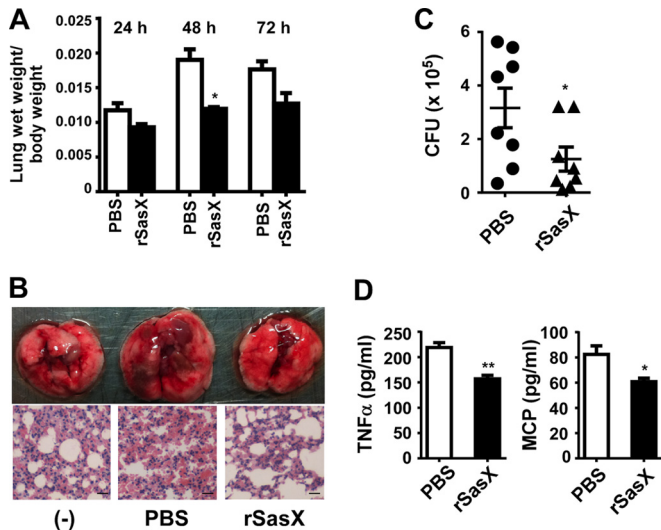
FIG 4 Active immunization with SasX reduces acute lung injury caused by MRSA ST239 in a lung infection model. Mice were challenged by introducing 10^9 live MRSA HS770 CFU into the nares 1 week after the first boosting with rSasX (or the PBS control). At 24, 48, and 72 h after inoculation, groups of mice were euthanized. (A) Lung wet weight/body weight ratios. *, $P < 0.05$. (B) The left lungs of mice euthanized at 48 h after infection were fixed in 4% formalin for H&E staining. Scale bars, 50 μm. (C and D) The right lungs of mice euthanized at 48 h after infection were homogenized, and the homogenized lung tissue was used for CFU (C) and cytokine detection (D). *, $P < 0.05$; **, $P < 0.01$.
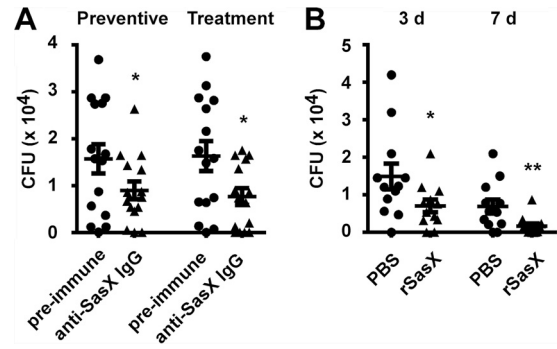

FIG 5 Active or passive immunization with SasX reduces nasal colonization caused by MRSA ST239 in a nasal colonization model. (A) Passive immunization. Mice received a total of 10 mg IgG of either rabbit preimmune serum or polyclonal anti-SasX IgG in 24-h intervals via intraperitoneal injection 24 h before (preventive effect) or after (treatment effect) nasal colonization with 10^7 CFU of MRSA strain HS770. At 7 days after inoculation, all mice were euthanized and evaluated for nasal carriage of *S. aureus*. Numbers are total CFU per nose. *, $P < 0.05$. (B) Active immunization. Mice were challenged by introducing 10^7 live bacterial cells of MRSA strain HS770 into the nares 1 week after the first boosting with rSasX (or the PBS control). Three days and 7 days after inoculation, 12 mice per group were euthanized and evaluated for nasal carriage of *S. aureus*. Numbers are total CFU per nose. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

S. aureus vaccine development is aimed at reducing the severity of *S. aureus* infections via two possible routes. Vaccines may reduce pathogen virulence during infection and allow the immune system to clear off the infection. Alternatively, *S. aureus* vaccines may reduce colonization by *S. aureus*, which is linked to subsequent infection (4, 20). In addition to fulfilling common prerequisites for optimal vaccine targets, such as accessibility on the surface of *S. aureus*, the SasX protein has been demonstrated to significantly impact both *S. aureus* nasal colonization and *in vivo* virulence in major *S. aureus* infection types (10). It is thus a potential target for both anti-*S. aureus* vaccination strategies aimed at colonization and/or infection.

Only a limited number of virulence determinants in *S. aureus* are widely distributed among virtually all *S. aureus* strains, such as alpha-toxin or phenol-soluble modulins (17). In ongoing efforts to use these as vaccination targets, alpha-toxin toxoid, for example, has proven to represent a very promising vaccination agent in efforts to reduce *S. aureus* skin and lung infection (21). In addition, we believe that virulence determinants that are found to strongly impact colonization and virulence phenotypes in MRSA strains with considerable epidemiological importance, wide geographical distribution, and likely further spread, such as SasX, should also be analyzed as potential vaccine targets. Other surface proteins, such as ClfA or IsdA, which impact nasal colonization in animal models, have previously shown success as immunogens in experimental animal colonization studies (22–24). For these reasons, we here analyzed whether active and passive immunization with SasX as the target reduces *S. aureus* skin and lung infection and has the potential to reduce *S. aureus* nasal colonization.

We found that SasX was strongly immunogenic in mice with a dominant production of IgG1 subtype antibodies. This suggests a Th2-dominant response. The induction of a high proportion of IgG1 is a typical feature of protein immunization (25). In addition, IgG1 was previously shown to be the most efficient opsonization isotype for neutrophils (26); therefore, the higher titers of total IgG and the dominant IgG1 subtype might be responsible for the superior protection conferred by SasX immunization than by immunization with inactivated *S. aureus*.

In our study, active immunization with SasX significantly reduced acute skin and lung infection in mice, and passive immunization with IgG purified from a rabbit polyclonal antiserum developed against SasX also reduced acute skin infection. Furthermore, active or passive immunization reduced nasal colonization in mice, with passive immunization being successful in both treatment and prevention fashions. Moreover, anti-SasX antibodies promoted elimination of *sasX*-positive MRSA by human neutrophils. These results suggest that SasX could be a valuable component in vaccine strategies targeting epidemic *S. aureus* hospital MRSA infections in Asia, among which the SasX protein is frequently expressed. Nowadays, such strategies are commonly set up to develop a multivalent vaccine aimed at a selection of targets with predominant effects on virulence and/or colonization (27). Furthermore, our present results underscore our previous findings indicating a significant impact of SasX on acute types of *S. aureus* infection as well as nasal colonization (10).

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