Abscess Formation and Alpha-Hemolysin Induced Toxicity in a Mouse Model of *Staphylococcus aureus* Peritoneal Infection

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*Staphylococcus aureus* is a frequent cause of skin infection and sepsis in humans. Preclinical vaccine studies with *S. aureus* have used a mouse model with intraperitoneal challenge and survival determination as a measure for efficacy. To appreciate the selection of protective antigens in this model, we sought to characterize the pathological attributes of *S. aureus* infection in the peritoneal cavity. Testing C57BL/6J and BALB/c mice, >10⁶ CFU of *S. aureus* Newman were needed to produce a lethal outcome in 90% of animals infected via intraperitoneal injection. Both necropsy and histopathology revealed the presence of intraperitoneal abscesses in the vicinity of inoculation sites. Abscesses were comprised of fibrin as well as collagen deposits and immune cells with staphylococci replicating at the center of these lesions. Animals that succumbed to challenge harbored staphylococci in abscess lesions and in blood. The establishment of lethal infections, but not the development of intraperitoneal abscesses, was dependent on *S. aureus* expression of alpha-hemolysin (Hla). Active immunization with nontoxicogenic Hla₃₃₃₅₅₃ or passive immunization with neutralizing monoclonal antibodies protected mice against early lethal events associated with intraperitoneal *S. aureus* infection but did not affect the establishment of abscess lesions. These results characterize a mouse model for the study of intraperitoneal abscess formation by *S. aureus*, a disease that occurs frequently in humans undergoing continuous ambulatory peritoneal dialysis for end-stage renal disease.

*S. aureus* is a commensal of the human skin and nares (45). When local or systemic host defenses are breached, *S. aureus* can cause a wide variety of disease manifestations ranging from skin and soft tissue infections (SSTI) to bacteremia, sepsis, endocarditis, and pneumonia (36). Many *S. aureus* strains have acquired multiple antibiotic resistance traits and are designated MRSA (methicillin-resistant *S. aureus*) (16). Treatment of complicated SSTI with MRSA typically requires hospital admission and intravenous administration of vancomycin, daptomycin, linezolid, or clindamycin (35). In 2007, *S. aureus* infection occurred in 4% of admissions to U.S. hospitals (32). The overall mortality associated with *S. aureus* infection is greater than that of any other infectious disease in the United States (31). A U.S. Food and Drug Administration-licensed vaccine that prevents *S. aureus* diseases is currently not available (15).

*S. aureus* colonization represents an infectious disease threat for immunocompromised individuals and patients with chronic disease states (36). End-stage renal disease (ESRD) affects 0.17% of the United States population, requiring the provision of either hemodialysis or peritoneal dialysis as a substitute for impaired kidney function. Central venous catheterization is performed in support of hemodialysis. In ESRD patients colonized with *S. aureus*, indwelling catheters are often associated with infection at the catheter insertion sites and bacteremia, in particular in patients with diabetes (22, 54). As an alternative to hemodialysis, ESRD patients may be offered continuous ambulatory peritoneal dialysis (CAPD). Peritoneal dialysis catheters form a nidus for staphylococcal infection at the skin insertion site, along the length of the tunneled device, and often are associated with peritonitis (37). Even with hospital admission and expert clinical care, staphylococcal peritonitis may either persist or reoccur and is therefore associated with significant mortality (51). CAPD, in spite of its technical convenience and decreased cost, is offered to only a select group of ESRD patients (7%) trained to perform aseptic dialysis at home and willing to assume the risk of bacterial peritonitis (30).

Several mouse models have been developed as surrogates for the study of *S. aureus* infections that occur in humans. These models include but are not limited to intravenous challenge with staphylococci to induce sepsis (12, 29) or endocarditis (44), subcutaneous injection of staphylococci to generate skin and soft tissue infections (10, 52), and intranasal instillation of staphylococci to induce pneumonia (5). The identification of discrete, disease-specific virulence factors of *S. aureus* in each of these models underscores the versatility of the pathogen (8, 9, 11). This work has led to the appreciation that preventive strategies may have to include multiple vaccine antigens to address different staphylococcal diseases (15). An animal model that characterizes the pathogenesis and virulence factors for *S. aureus* peritonitis has thus far not been established.

Because of the technical ease of infecting animals via intraperitoneal injection, early work on the pathogenesis of *S. aureus* infections used this route of challenge to infect mice (13, 33). These studies led to the characterization of *S. aureus* hemolytic activity as a contributory factor to lethal disease (14, 25, 34). Alpha-hemolysin, a secreted pore-forming toxin that utilizes its receptor ADAM10 to disrupt cellular membranes and alter the integrity of...
the epithelial barrier (24, 53), kills mice when the purified protein (10 μg or more) is injected into the peritoneal cavity (4). Expression of plasmid encoded hla antisense in S. aureus, which was shown to reduce the synthesis of alpha-hemolysin, was found to diminish the virulence of the staphylococcal strain in the intraperitoneal challenge model (27). Recombinant HlaH35L, a nontoxicogenic variant of alpha-hemolysin (42), was used to elicit toxin-neutralizing antibodies in rabbits; passive transfer of these antibodies into mice prevented the lethal outcome of intraperitoneal S. aureus challenge (41).

Intraperitoneal challenge has been utilized in studies examining vaccine efficacy, owing to the ability to easily deliver large inocula and generate highly reproducible data in lethal dose challenge experiments (20, 40, 41, 50). It has been assumed that staphylococcal injection into the peritoneum causes a rapidly fatal spread of these microbes into the bloodstream (13, 33). Of note, the number of staphylococci required to produce lethal disease is at least 10-fold higher for intraperitoneal versus intravenous challenge (12), suggesting that the lethal outcome of peritoneal challenge may be governed by factors different from those identified for sepsis after intravenous challenge (39).

We sought here to elucidate the disease progression associated with S. aureus injection into the peritoneal cavity of mice. As the route of inoculation mimics the infectious process of CAPD-associated S. aureus peritonitis, the characterization of pathological features for staphylococcal peritonitis in mice may enable the characterization of specific protective antigens. If so, this model may be useful for the development of a vaccine that can prevent staphylococcal peritonitis and protect ESRD patients who depend on CAPD for dialysis and survival.

MATERIALS AND METHODS

Animal care and compliance statement. All experiments involving the care and use of animals followed protocols that were reviewed, approved and performed under the regulatory supervision of The University of Chicago’s Institutional Biosafety Committee and the Institutional Animal Care and Use Committee. Animal care was managed by The University of Chicago Animal Resource Center, accredited by the American Association for Accreditation of Laboratory Animal Care and the Department of Health and Human Services (DHHS; number A3523-01). Animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the DHHS Guide for the Care and Use of Laboratory Animals. Veterinary Care was under the direction of full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine. BALB/c (strain code 028) and C57BL/6J (stock number 000664) mice were purchased from Charles River and Jackson Laboratories, respectively.

Statistical analysis. Mouse survival was analyzed for significance using the two-tailed log-rank test. The bacterial load following S. aureus infection with wild-type and/or isogenic mutant strains, represented as the log_{10} CFU ml^{-1} in peritoneal lavage fluid or blood and the log_{10} CFU/g of organ tissue, was analyzed with the Mann–Whitney test for statistical significance. Quantification of mouse abscess formation was analyzed for statistical significance using the unpaired two-tailed Student t test. Statistical analyses were performed using GraphPad Prism 4 software. All experiments were examined for reproducibility.

Bacterial strains, media, and growth conditions. Mutants harboring the bursa aurelis mariner transposon were obtained from the Phoenix (ΦN2) library and were transduced with bacteriophage φ85 into S. aureus Newman as described previously (2). S. aureus cultures were grown at 37°C in tryptic soy broth (TSB), and ΦN2 transposon mutant cultures were supplemented with 10 μg of erythromycin ml^{-1}. Strains MW2 and USA300 are clinical isolates that have been described previously (18, 21).

E. coli strain BL21(DE3) was grown in Luria-Bertani (LB) broth containing 100 μg of ampicillin ml^{-1} at 37°C when plasmid was present.

**Protein purification.** E. coli BL21(DE3) harboring the expression vector pGEX-6p-1 hla(H35L), described previously (9), was diluted 1:10, grown at 37°C for 2 h, followed by induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). At 5 hours after induction, the cells were harvested by centrifugation at 7,000 × g for 10 min, bacterial sediment suspended in phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.0]) and lysed in a French pressure cell at 14,000 lb/in². Lysates were cleared by ultracentrifugation at 45,000 × g for 45 min, and the supernatants were applied to a glutathione-Sepharose column equilibrated with PBS. Glutathione beads were washed with 10 column volumes of PBS, followed by 10 column volumes of cleavage buffer (250 mM Tris [pH 6.8], 750 mM NaCl, 5 mM EDTA, 5 mM diithiothreitol [DTT]). GST-HlaH35L was incubated with 100 U of PreScission protease (GE Healthcare) in cleavage buffer for 5 h at 4°C and eluted from the column with the same buffer. To remove endotoxin, 1% Triton X-114 was added, and the solution was cooled on ice for 10 min, incubated at 37°C for 10 min and centrifuged at 16,000 × g for 10 min. This was repeated twice and resulting supernatant was loaded onto a Hi-Trap desalting column (GE Healthcare) and eluted in endotoxin-free PBS (Cellgro). Concentration and purity were assessed by the bicinchoninic acid method (BCA; Pierce) and SDS-PAGE analysis.

**Immunization studies.** For active immunizations, 3-week-old BALB/c mice were injected intramuscularly with 20 μg of HlaH35L recombinant protein emulsified in 100 μl of complete Freund adjuvant (Difco), followed by a boost on day 12 with 20 μg of HlaH35L protein emulsified in incomplete Freund adjuvant (Difco). Blood was collected on day 20 via periorbital vein puncture with heparinized micro-hematocrit capillary tubes (Fisher). Z-gel serum separation microtubes (Sarstedt) were used to collect serum antibodies, and specific antibody production was assessed via enzyme-linked immunosorbent assay (ELISA) as described previously (28). On day 21, mice were challenged by intraperitoneal injection with 5 × 10^6 CFU of S. aureus Newman.

For passive immunization studies, 6-week-old BALB/c mice were injected intraperitoneally with 5 mg of monoclonal antibody (MAB) 7B8 (48)/kg or IgG2a isotype control in a total volume of 200 μl. Murine IgG2a isotype control was purchased from R&D Systems, Inc., and MAb 7B8, which has been previously described (48), was generated against HlaH35L by the Frank W. Fitch Monoclonal Antibody Facility at the University of Chicago. Mice were infected 4 h after immunization by intraperitoneal injection with 5 × 10^6 CFU of S. aureus Newman.

**Mouse intraperitoneal challenge.** Overnight cultures of S. aureus were inoculated 1:100 into fresh TSB and grown for 2 h with shaking at 37°C. Staphylococci were sedimented, washed, and suspended in sterile PBS to obtain an inoculum of 5 × 10^6 CFU ml^{-1}. Six-week-old female BALB/c mice were infected via intraperitoneal injection with 5 × 10^6 CFU of staphylococci. Inocula were determined by CFU enumeration following serial dilution, plating on tryptic soy agar, and growth at 37°C. Infected animals were monitored for morbidity or recovery over a period of 15 days.

**Peritoneal abscess formation.** At timed intervals after infection, mice were killed by CO₂ inhalation and subjected to necropsy, and a representative abscess located at or near the anterior abdominal wall was excised. Abscess samples were homogenized in 0.1% Triton X-100 using a high sheat lab homogenizer (Omni International) and homogenates serially diluted, plated, and incubated for CFU enumeration. Abscess samples from each cohort of mice werefixed in 10% formalin for 24 h at room temperature, embedded, thin-sectioned, stained with hematoxylin and eosin (H&E) or Masson's trichrome and examined by light microscopy.

**Renal abscess formation.** Inocula were prepared as described above and suspended in PBS to a final concentration of 10^6 CFU ml^{-1}. Six-week-old female BALB/c mice were anesthetized by intraperitoneal injection with a cocktail of 100 mg of ketamine/ml and 2 mg of xylazine/ml per kilogram of body weight, followed by infection with 100 μl of bacterial
suspension (10⁷ CFU) by retro-orbital injection. On day 4 after infection, the mice were killed by CO₂ inhalation, and the left kidneys were excised and homogenized in 0.01% Triton X-100 as described for abdominal wall tissue. Homogenates were serially diluted, plated, and incubated at 37°C for CFU enumeration. The remaining kidneys were fixed in 10% formalin for 24 h at room temperature. Tissues were embedded in paraffin, sectioned, stained with H&E, and examined by light microscopy.

**Immunohistochemistry.** Immunohistochemistry staining was performed at the Human Tissue Resource Center at The University of Chicago. Briefly, abscess samples were deparaffinized and rehydrated through xylene and serial dilutions of ethanol to distilled water. Samples were incubated in Dako antigen retrieval buffer (pH 6.0) and heated in a steamer oven at 96°C for 20 min. After rinsing, the slides were incubated in 3% hydrogen peroxide for 5 min and then 10% normal serum in PBS with 0.025% Triton X-100 for 30 min. Next, 10% human IgG was used as a blocking reagent for 30 min. Primary antibody (1:500 rat anti-mouse F4/80 [MCAP497; Serotec] or 1:500 rabbit anti-mouse fibrinogen [Innovative Research]) was applied to the slides for overnight incubation at 4°C in a humidity chamber. After three washes with Tris-buffered saline, the slides were incubated with biotinylated secondary antibody (1:50 biotinylated anti-rat IgG [BA-4001; Vector Laboratories] or 1:200 biotinylated anti-rabbit IgG [BA-1000; Vector Laboratories]). Antigen-antibody binding was detected with DAB chromogen. The slides were briefly immersed in hematoxylin for counterstaining and evaluated by light microscopy.

**RESULTS**

**Challenge of mice by intraperitoneal injection of S. aureus.** Cohorts of BALB/c mice were infected via intraperitoneal injection with S. aureus Newman (1), a human clinical isolate that has been used for animal model development (11, 19). Injection of 2 × 10⁸ CFU of S. aureus Newman resulted in 0 to 25% mortality over 15 days (Fig. 1A). Increasing the inoculum to 5 × 10⁸ to 7 × 10⁸ CFU of S. aureus Newman caused 50 to 70% of infected mice to succumb to infection; most of these animals developed a lethal infection within 24 h (Fig. 1A). An inoculum of 6 × 10⁹ CFU of S. aureus Newman caused 95% mortality, with all deaths occurring within the first 12 h of challenge (Fig. 1A). Similar results were obtained when S. aureus Newman was injected into the peritoneal cavity of C57BL/6J mice, indicating that the lethal outcome to staphylococcal challenge is not mouse strain specific (Fig. 1B). In agreement with earlier studies (34), a lethal outcome of mouse experiments was dependent on metabolically active, replicating staphylococci, since all animals injected with heat-killed staphylococci survived the intraperitoneal challenge (Fig. 1C).

**Fate of staphylococci inoculated into the peritoneal cavities of mice.** Inoculation into the peritoneal cavity may enable S. aureus to gain access to the bloodstream. For example, this could occur through staphylococcal entry into capillaries of the parietal peritoneum lining the inside of the abdominal wall, which drain into general circulation. Alternatively, staphylococci could enter celiac or mesenteric capillaries within the visceral peritoneum, which drain into the portal vein. To test whether intraperitoneal inoculation leads to the dissemination of S. aureus into the bloodstream, infected mice were subjected to cardiac puncture at timed intervals after challenge. Blood samples were spread on agar plates, and bacterial loads were enumerated as CFU counts. After the injection of 5 × 10⁸ CFU of S. aureus Newman, 3 ± 0.5 log₁₀ CFU of staphylococci ml⁻¹ were detected in blood as early as 3 h postinjection (Fig. 2A). S. aureus CFU in blood decreased over time and, by 2 days postinfection, staphylococci could no longer be detected (Fig. 2A).

To further monitor the fate of staphylococci after challenge, the peritoneal cavity was rinsed with 1 ml of PBS. Bacterial load in lavage fluids was determined by counting S. aureus CFU on agar plates (Fig. 2B). At 3 h after inoculation into the peritoneal cavity, 5 ± 0.05 log₁₀ CFU ml⁻¹ were recovered from the lavage fluid (Fig. 2B). Within 24 h, the number of S. aureus in the lavage fluid had declined to 3 ± 0.59 log₁₀ CFU ml⁻¹. Eventually, staphylococci could no longer be isolated from peritoneal lavage fluids of infected animals (Fig. 2B).

To investigate whether injected staphylococci are either sequestered within the peritoneal cavity or cleared by the immune system, infected animals were subjected to necropsy at timed intervals after challenge. As early as 24 h after the injection of S. aureus into the peritoneal cavity, we observed round or oval yellow lesions on the abdominal wall in close proximity to the inoculation site (Fig. 2C), which were not detected in animals inoculated with heat-killed bacteria. Abdominal wall tissue at the injection site was excised 24 h postinfection, homogenized, and...
spread onto agar medium. *S. aureus* Newman (8 ± 0.14 log₁₀ CFU) was isolated from tissue homogenates.

During necropsy, the peritoneal lining cannot be separated from abdominal organs such as kidney, liver, or spleen. Thus, the removal of organs from the abdominal cavity of mice and the enumeration of staphylococci in tissue homogenates derived from these organs cannot precisely delineate the intra- or retroperitoneal position of staphylococcal lesions. Nevertheless, histopathologic features of staphylococcal disease in the abdominal cavity do reveal the intra- or extraperitoneal location of these lesions (Fig. 3). Histopathologic analysis of kidneys from animals infected for 24 h revealed the appearance of abscess lesions on 12.5% of organ surfaces but did not identify parenchymal abscess lesions (Fig. 3A). The number of kidneys with surface abscesses increased to 25% by 48 h, and similar numbers of surface abscesses were enumerated during subsequent time intervals (Fig. 3A). On day 3, we observed parenchymal abscess lesions in kidneys from 22.5% of animals that had been infected via intraperitoneal challenge (Fig. 3A). These lesions were indistinguishable from abscesses formed after intravenous challenge with 5 × 10⁷ CFU of *S. aureus* Newman, which elicits ~4 lesions per organ tissue (11). Histopathology analyses confirmed that surface abscesses were associated with the peritoneal lining but did not affect the kidney parenchyma (Fig. 3B and C). Together, these findings suggest that the mortality associated with intraperitoneal injection of *S. aureus* Newman into mice is not caused by bacterial entry into the bloodstream or the massive dissemination of staphylococci into organ systems. Furthermore, most of the staphylococci injected into the peritoneal cavity are sequestered within lesions on the abdominal wall.

**Fibrin and collagen deposits sequester staphylococci within the peritoneal cavity.** Peritoneal lesions that had formed within 24 h of infection were excised during necropsy. Tissues were fixed in formalin, thin sectioned, and stained with Masson's trichrome stain. When viewed by light microscopy, round or oval lesions were detected on the abdominal wall with a diameter of 1.325 ± 0.65 mm (Fig. 4A). Trichrome staining revealed the lining of the parietal peritoneum (blue) onto which each lesion was immobilized. Lesions were surrounded by a layer (16.8 ± 1.2 μm in diameter) of amorphous, red-stained material (Fig. 4B). The molecular composition of the outermost layer was characterized as fibrin deposits, since this amorphous material was immunoreactive to fibrinogen–specific antibodies (Fig. 4C and D). Within lesions, large numbers of staphylococci and surrounding immune

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**FIG 2** Fate of staphylococci in the peritoneal cavities of mice. Six-week-old BALB/c mice were infected by intraperitoneal injection with 5 × 10⁶ CFU of *S. aureus* Newman. (A) At timed intervals (3 h or 1, 2, 3, 6, or 10 days) after inoculation, blood from cohorts of mice (n = 7) was collected by cardiac puncture and plated on tryptic soy agar (TSA), and the staphylococcal load was enumerated as CFU. Black bars indicate means of observations. (B) At timed intervals (3 h or 1, 3, 6, 10, or 15 days) after inoculation, the peritoneal cavity of mice was rinsed with 1 ml of sterile PBS, and the staphylococcal load was enumerated as CFU on agar plates. (C) Animals that had been infected by intraperitoneal injection with 5 × 10⁶ CFU of *S. aureus* Newman were euthanized and necropsied, and peritoneal abscesses on the abdominal walls (black arrowheads) were excised and analyzed for histopathology or staphylococcal load (see subsequent figures).

**FIG 3** Staphylococcal lesions associated with the kidney. (A) Six-week-old BALB/c mice were infected by intraperitoneal injection with 5 × 10⁶ CFU of *S. aureus* Newman. At timed intervals, animals (n = 15) were euthanized and necropsied, and the kidneys were excised, fixed in formalin, thin sectioned, and analyzed for histopathology of H&E-stained tissues. The percentages of kidneys with surface abscesses or parenchymal abscesses were recorded. (B and C) Example of an H&E-stained surface abscess associated with the peritoneal lining of the kidney. (D and E) Example of an H&E-stained abscess positioned within the kidney parenchyma.
cells were detected (Fig. 4A and C). Lesions from animals that were infected for 3 days displayed an increase in size (2.426 ± 0.271 mm in diameter) and in the diameter of the amorphous outer layer (55.2 ± 26.5 μm in diameter) (Fig. 4E and F). Trichrome staining revealed collagen deposits in the outer layer of day 3, but not day 1 lesions (Fig. 4E and F). Compared to the outer fibrin layer, collagen deposits were immediately juxtaposed to a second, inner layer of fibrin (Fig. 4G and H). Large numbers of staphylococci were located at the center of each lesion and surrounded by immune cells (Fig. 4E and G).

Persistence of *S. aureus* lesions in the peritoneal cavity. At timed intervals after intraperitoneal inoculation with $5 \times 10^6$ CFU of *S. aureus* Newman, mice were euthanized. Lesions were excised during necropsy, fixed with formalin, thin sectioned, and stained with H&E. Twenty-four-hour-old lesions were comprised of large clusters of staphylococci surrounded by small amounts of fibrin and massive deposits of immune cells, most of which appeared to be necrotic (Fig. 5A to C). On day 3, the size of the lesions had increased. At the center, fibrin deposits and immune cells surrounding a nidus of bacteria were detected (Fig. 5D to F). By day 6, the lesions had increased further, and their architecture was altered to reveal more extensive infiltrations of immune cells and a concomitant decrease in the size of the staphylococcal community (Fig. 5G to I). By day 15, staphylococcal communities were clearly delineated by a surrounding eosinophilic pseudocapsule and often positioned at the periphery of the lesion (Fig. 5J to L). The pseudocapsule of staphylococci is comprised of fibrin deposits, as revealed by staining with fibrinogen-specific antibodies (see Fig. 4E and G).
A layer of macrophages, detected with antibodies specific for the F4/80 surface marker, was positioned on the inside of the outer layers of day 15 lesions, immediately adjacent to collagen deposits (Fig. 6A and C). Macrophages were also found interspersed throughout the center of the lesion, which harbors large numbers of polymorphonuclear leukocytes (Fig. 6A and C). Most leukocytes of day 15 lesions had undergone apoptosis, as evidenced by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling)-positive staining (Fig. 6E and G). Of note, immune cells surrounding the staphylococcal communities in day 15 lesions were not TUNEL positive (Fig. 6E and G). Together, these data indicate that the deposition of fibrin surrounding staphylococci in the peritoneal cavity leads to their sequestration into a defined lesion that is further modified by massive infiltration of immune cells. At least for the first 3 days, infiltrating immune cells appear to be predominantly granulocytes and macrophages.

Staphylococcal genes required for the pathogenesis of intraperitoneal infections. Sortase A (SrtA), a transpeptidase, anchors surface proteins with an LPXTG-motif sorting signal to the cell wall envelope (38). Deletion of the srtA gene abolishes the ability of staphylococci to seed abscesses in organ tissues or to cause lethal sepsis after intravenous challenge of mice (11, 29). When injected into the peritoneal cavities of mice, the srtA mutation caused a moderate reduction in animal deaths within the first 24 h compared to wild-type S. aureus Newman (Fig. 7A). We also tested variants with mutational lesions in regulatory genes. A mutant with an insertional lesion in the global regulator mgrA, which encodes a transcriptional regulator of genes responding to oxidative stress, displayed reduced mortality (Fig. 7A). Moreover, S. aureus variants unable to express the two-component virulence regulators saeR and agrA did not produce a lethal outcome to staphylococcal injection into the peritoneal cavity over the course of the entire experiment (Fig. 7A).

The agr locus encodes a quorum-sensing (QS) system that controls the expression of extracellular proteins (43). The sae locus governs a complex transcriptional pattern including the agr genes and thereby contributes to the control of exoprotein expression (43). The best-characterized virulence factor of the agr and sae regulons is alpha-hemolysin (Hla), a toxin that is essential for the
pathogenesis and lethal outcome of *S. aureus* pneumonia in mice (6). Hla has been implicated as an important virulence determinant for staphylococcal peritonitis (41). However, to the best of our knowledge, *S. aureus hla* mutants have never been examined in the peritoneal challenge model. To address this question, mice were injected with $5 \times 10^8$ CFU of *S. aureus* Newman, its *hla* mutant, or the *hla* mutant harboring a plasmid for the expression of wild-type *hla* (*p* *hla*). Most animals that were infected with the wild-type parent strain succumbed to infection within 48 h, whereas 80% of animals challenged with the *S. aureus* *hla* mutant survived the challenge (Fig. 7B). Of note, most of the animals that were infected with the *hla* mutant survived for the first 48 h, the time interval when lethal disease is attributable to the secreted toxin; however, some of the surviving animals died over the 15-day course of the experiment (Fig. 7B). These results indicate that not all animal mortality during peritoneal infection model is caused by alpha-hemolysin secretion (Fig. 7B). Plasmid-encoded *hla* complemented the virulence defect of the *hla* mutant strain, since most of the infected animals died within the first 24 h (Fig. 7B).

Synthesis and secretion of *agr*-regulated alpha-hemolysin is a variable trait of epidemic *S. aureus* clones and strains harboring a single nucleotide polymorphism in *hla* that introduces a premature STOP are still capable of causing human disease (17). MRSA strain USA300 LAC, which is responsible for the current epidemic of community-acquired infections, secretes more alpha-hemolysin than *S. aureus* Newman (6). In contrast, MRSA MW2, a clinical isolate associated with skin and soft tissue infections, expresses much less toxin than either Newman or USA300 LAC (6). In agreement with the overall concept that Hla is a key determinant for the early lethal outcome of peritoneal infection in mice, all

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**FIG 6** Macrophages and apoptotic immune cells in peritoneal abscesses caused by *S. aureus*. BALB/c mice were infected by intraperitoneal injection with $5 \times 10^8$ CFU of *S. aureus* Newman wild-type or *hla* mutant bacteria. Animals were euthanized 15 days after infection and necropsied, and peritoneal abscesses on the abdominal wall were excised, fixed in formalin, thin sectioned, and processed for histopathology. (A to D) Murine macrophages in peritoneal abscesses were detected in serial thin sections with an antibody against F4/80. (E to H) Apoptotic immune cells were identified by using TUNEL. Arrowheads identify staphylococci (yellow), the pseudocapsule of staphylococcal abscess communities (white), macrophages (green), and apoptotic cells (red).
animals that received $5 \times 10^8$ CFU of S. aureus MW2 survived the challenge, whereas most of the animals that received an equal dose of S. aureus Newman or USA300 did not (Fig. 7C).

**Alpha-hemolysin is dispensable for S. aureus peritonitis or renal abscess lesions.** S. aureus Newman or its isogenic hla mutant ($5 \times 10^8$ CFU) were injected into the peritoneal cavity of BALB/c mice ($n = 10$). Animals were euthanized 6 or 15 days after challenge, necropsied, and examined for lesions on the abdominal wall. Most infected animals harbored yellow lesions on the abdominal wall, irrespective of whether challenge occurred with the wild-type or hla mutant strain (Fig. 8A). Lesions were excised, and tissues were homogenized and plated on agar to enumerate the bacterial load as CFU. The numbers of staphylococci retrieved from wild-type Newman or hla mutant lesions were not significantly different (Fig. 8B). Lesions that were excised during necropsy on day 6 or 15 after challenge were also fixed in formalin.
thin sectioned, and stained with H&E. The overall size and histopathological attributes (staphylococcal communities, fibrin and collagen deposits, and immune cell infiltrates) of lesions caused by wild-type and hla mutant staphylococci were indistinguishable (Fig. 9). Further, the distribution of macrophages and apoptotic immune cells in lesions formed by wild-type and hla mutant S. aureus Newman were also indistinguishable (Fig. 6B, D, F, and H). To test whether alpha-hemolysin is dispensable also for the establishment of abscess lesions in organ tissues, staphylococci were injected intravenously into the retroorbital plexi of BALB/c mice (11). At 5 days after challenge, the animals were euthanized, and abscess formation in renal tissues was analyzed by histopathology and staphylococcal load in homogenized tissues. H&E-stained tissues revealed similar numbers of lesions and pathological features of abscesses that were caused by either wild-type S. aureus Newman or the hla mutant strain (11). Further, the staphylococcal loads within renal abscess lesions were similar for wild-type, hla mutant, or complemented mutant strains (Fig. 8C).

**Hla-neutralizing antibodies do not protect against staphylococcal abscess lesions.** Immunization of mice with the nontoxicogenic variant HlaH35L raises toxin-neutralizing antibodies that protect animals against lethal staphylococcal pneumonia (intranasal challenge), lethal S. aureus sepsis (intravenous challenge), staphylococcal dermonecrosis (subcutaneous challenge), and lethal intraperitoneal challenge (9, 26, 41, 47). We sought to determine whether Hla-neutralizing antibodies can also protect animals against staphylococcal peritonitis. After immunization of mice with a prime-boost regimen of HlaH35L or a PBS mock control, the mice were challenged by intraperitoneal injection with 5 × 10⁸ CFU of S. aureus Newman. Compared to the PBS/mock control cohort, HlaH35L-immunized mice were protected from lethal disease (Fig. 10A). Further, passive transfer of MAb 7B8 (αHla), an MAb that neutralizes alpha-hemolysin (48), provided protection against early lethal disease after intraperitoneal injection of 5 × 10⁸ CFU of S. aureus Newman, whereas IgG2a isotype control antibodies did not (Fig. 10B). Actively or passively immu-
nized animals were necropsied on day 15 after intraperitoneal challenge, and abscess formation was analyzed by inspection of the abdominal wall and histopathology of thin-sectioned, H&E-stained tissues. Although antibodies against Hla provided protection from early lethal disease, immunized animals were not protected from staphylococcal abscess formation, since similar numbers of abdominal wall lesions and similar histopathology features of staphylococcal lesions were detected in Hla\textsuperscript{H35L\textsuperscript{+}} or αHla-immunized and control cohorts (Fig. 10C and D).

**DISCUSSION**

Preclinical vaccine studies have used intraperitoneal challenge of mice with *S. aureus* as a model for the protection of humans against staphylococcal disease (20, 41). Earlier work on this model suggested that alpha-hemolysin, a pore-forming toxin secreted by many but not all *S. aureus* strains (17), is an important virulence factor (27). Further, antibodies raised against the nontoxigenic variant Hla\textsuperscript{H35L} protected mice against the lethal outcome of intraperitoneal challenge with *S. aureus* (41). Hla\textsuperscript{H35L} cannot be the sole component of a vaccine that aims to protect against a wide spectrum of staphylococcal diseases, since toxin-neutralizing antibodies do not provide full protection against skin abscess formation after subcutaneous inoculation or modify the development of abscesses in internal organs after intravenous inoculation of mice (7, 9, 26). If so, what are the pathological events associated with peritoneal infection of *S. aureus* and are they confined to early lethality after inoculation with a large dose of staphylococci? To address these questions, we provide an analysis of mice with intraperitoneal *S. aureus* infection, examining the clinicopathologic features of disease.

We confirm that hla encodes a determinant of early lethal outcomes associated with intraperitoneal *S. aureus* challenge. We report that hla knockout mutants cause fewer lethal events, similar to hla antisense mutants (27), and that this phenotype was complemented in trans by wild-type hla. Further, passive transfer of neutralizing MAb 7B8 (48) protected against Hla-mediated lethal disease. Nevertheless, both hla mutants and animals immunized with MAb 7B8 (αHla) and challenged with wild-type *S. aureus* were able to seed peritoneal abscess lesions that persisted for the duration of the entire experiment, similar to animals that had been infected with wild-type *S. aureus* strains.

Peritoneal infections with *S. aureus* occur frequently in patients undergoing CAPD (49) and manifest as inflammation of the peritoneal catheter exit site and tunnel, peritonitis as well as peritoneal abscesses (46, 55). Although the development of flush-and-fill CAPD catheters and procedural changes have reduced the incidence of peritoneal infections in coagulase-negative staphylococci (23), *S. aureus* disease in CAPD patients has not declined and represents a life-threatening event requiring hospital admission and initiation of hemodialysis (49). Nevertheless, hemodialysis patients are also at increased risk for *S. aureus* sepsis and pneumonia (55). Thus, there is an urgent need to develop preventive measures and vaccines against *S. aureus* infections in patients with diabetes and ESRD, since the incidence of these co-morbid diseases is increasing in both developed and developing countries (3).

To begin to address the requirements for a staphylococcal vaccine that can prevent peritonitis, we used a mouse model of intraperitoneal *S. aureus* challenge and histopathology to study the development of peritoneal abscess lesions. The early mortality ob-
served with intraperitoneal S. aureus challenge is not associated with the formation of abdominal wall lesions, as demonstrated through our studies of S. aureus that lacks expression of alpha-hemolysin. In all of the cases examined, staphylococci were sequestered within the peritoneal cavity through early fibrin deposits and the subsequent formation of an outer layer of collagen. The continuous infiltration of immune cells, i.e., mostly granulocytes and macrophages, into lesions caused an increase in sizes of these peritoneal abscesses. During the 15-day observation period, infected mice were unable to clear abscesses or the staphylococcal communities within these lesions. Further, the histopathology of the lesions changed over time, as staphylococci were organized into communities with surrounding immune cells near the periphery of the lesions. We surmise, but do not yet know, that the peritoneal lesions may rupture over time and give rise to new lesions, as has been observed during human peritonitis and peritoneal abscesses (49). Revealing the histopathology of S. aureus abscess lesions in mice may now enable a search for staphylococcal genes that promote pathogen survival and replication in these lesions. A similar approach has previously been applied to the search for staphylococcal abscess formation following the intravenous challenge of mice or staphylococcal pneumonia (5, 11). Secreted gene products that were required for disease establishment could then be tested for vaccine development and the ability to prevent the infectious process (9, 12, 28, 29). Similar strategies may be used toward the identification of protective antigens that can prevent S. aureus peritonitis and peritoneal abscesses in patients with CAPD.

ACKNOWLEDGMENTS

We thank members of our laboratory for helpful discussions. This study was supported by grants from the National Institute of Allergy and Infectious Diseases, Infectious Diseases Branch (AI52474, AI75258, and AI92711 to O.S. and D.M.M.), and by Novartis Vaccines and Diagnostics (Siena, Italy). D.M.M., J.B.W., and O.S. acknowledge membership within and support from the Region V Great Lakes Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Consortium (NIH award #U54-AI-057153).

We declare a conflict of interest as inventors of patent applications that are related to the development of Staphylococcus aureus vaccines and are currently under commercial license.

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