A Single B-Repeat of *Staphylococcus epidermidis* Accumulation-Associated Protein Induces Protective Immune Responses in an Experimental Biomaterial-Associated Infection Mouse Model

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Nosocomial infections are the fourth leading cause of morbidity and mortality in the United States, resulting in 2 million infections and ~100,000 deaths each year. More than 60% of these infections are associated with some type of biomedical device. *Staphylococcus epidermidis* is a commensal bacterium of the human skin and is the most common nosocomial pathogen infecting implanted medical devices, especially those in the cardiovascular system. *S. epidermidis* antibiotic resistance and biofilm formation on inert surfaces make these infections hard to treat. Accumulation-associated protein (Aap), a cell wall-anchored protein of *S. epidermidis*, is considered one of the most important proteins involved in the formation of *S. epidermidis* biofilm. A small recombinant protein vaccine comprising a single B-repeat domain (Brpt1.0) of *S. epidermidis* RP62A Aap was developed, and the vaccine’s efficacy was evaluated in vitro with a biofilm inhibition assay and in vivo in a murine model of biomaterial-associated infection. A high IgG antibody response against *S. epidermidis* RP62A was detected in the sera of the mice after two subcutaneous immunizations with Brpt1.0 coadministered with Freund’s adjuvant. Sera from Brpt1.0-immunized mice inhibited *in vitro* *S. epidermidis* RP62A biofilm formation in a dose-dependent pattern. After receiving two immunizations, each mouse was surgically implanted with a porous scaffold disk containing $5 \times 10^6$ CFU of *S. epidermidis* RP62A. Weight changes, inflammatory markers, and histological assay results after challenge with *S. epidermidis* indicated that the mice immunized with Brpt1.0 exhibited significantly higher resistance to *S. epidermidis* RP62A implant infection than the control mice. Day 8 postchallenge, there was a significantly lower number of bacteria in scaffold sections and surrounding tissues and a lower residual inflammatory response to the infected scaffold disks for the Brpt1.0-immunized mice than for the ovalbumin (Ova)-immunized mice.

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venting S. epidermidis biofilm infection. In fact, antiserum against Aap has been shown to prevent both polysaccharide-based (11) and proteinaceous (14, 15) biofilm formation by S. epidermidis. Murine monoclonal antibodies (MAbs) raised against Aap have also been shown to inhibit in vitro S. epidermidis biofilm formation by 66% using a single MAb and 79% to 87% using a mixture of two different MAbs (21). A single-chain diabody comprising the two paratopes, one from each MAb, reduced S. epidermidis biofilm formation by ~93% (21).

However, there have been no in vivo studies directly demonstrating the potential of Aap or G5 domains as antibiotic vaccines. In this study, we focused on a single Aap B-repeat (Brpt1.0), composed of a 78-aa G5 domain and a 50-aa spacer segment, and investigated its immunogenicity and protective efficacy in a mouse model of S. epidermidis RP62A biomaterial-associated infection. Our study indicated that subcutaneous immunization with recombinant Brpt1.0 protein induced significant Th2-dominant anti-S. epidermidis IgG antibody responses. Antisera were capable of inhibiting S. epidermidis RP62A biofilm formation in vitro in a dose-dependent manner. A mouse study further showed evidence that immunization with the Brpt1.0 protein successfully protected C57BL/6J mice against implant-based S. epidermidis infections.

MATERIALS AND METHODS

Plasmid and bacterial strains. Plasmid pET-21a (Novagen, USA) was used as an expression vector that contains an N-terminal T7 TAG sequence and a C-terminal His tag sequence. Escherichia coli DH5α and BL21 (DE3) were used for DNA cloning and protein production, respectively. E. coli strains were grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C, supplemented with 100 μg/ml of ampicillin when cells were chemically transformed with plasmids. For the animal challenge and in vitro biofilm inhibition assays, S. epidermidis RP62A (ATCC 35984) was grown in tryptic soy broth (TSB) or on TSB agar plates at 30°C.

PCR amplification of DNA. A 384-bp DNA fragment of a single Aap B-repeat domain, named brpt1.0, was PCR amplified from the S. epidermidis RP62A genome. The primers were designed based on the NCBI GenBank reference sequence of the S. epidermidis RP62A complete genome (accession no. CP000029.1), including a forward primer (5′-ggggATCCATgcaGgttatgttaggatc-3′) with a BamHI restriction enzyme site (capital letters) and a reverse primer (5′-atgGGGCgCGgcaacccatcattctc-3′) with a NotI site (capital letters). Amplification of the brpt1.0 DNA was achieved by using Pfu DNA polymerase (catalog no. 600153; Agilent Technologies). The PCR program consisted of 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min, and a final extension for 10 min at 72°C. The PCR product was examined by electrophoresis on a 1% agarose gel.

Brpt1.0 protein production and purification. The desired PCR fragment was purified using the QiAquick PCR purification kit (Qiagen) and cloned into plasmid pET21a between the BamHI and NotI restriction sites in frame with the sequence encoding six histidine residues at the C terminus (His tag). Recombinant plasmid pET21a-brpt1.0 was transformed into chemical-competent E. coli DH5α cells and confirmed by restriction endonuclease double digestion and sequencing of the DNA insert before transformation into E. coli BL21 (DE3) cells. BL21 cells carrying recombinant plasmid pET21a-brpt1.0 were grown in LB medium at 37°C with shaking (250 rpm) to an A600 of 1, and then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to induce protein expression via the lacZ promoter. After an additional 3 h of shaking incubation at 37°C, cells were harvested. To determine whether the produced protein of interest was soluble or in inclusion bodies, the harvested cells were washed and resuspended in Tris buffer (pH 8.0) and lysed by ultra-
sound sonication in bursts of 30 s on ice for 5 min. The lysed cells were centrifuged at 8,000 rpm for 10 min, and samples from the supernatant (soluble fraction) and the pellet (inclusion bodies) were separately analyzed by SDS-PAGE. We observed that the recombinant protein Brpt1.0 was located in the soluble fraction.

The soluble protein Brpt1.0 with a C-terminal His tag was purified by metal-chelate affinity chromatography (MCAC) using a B-PER 6×His fusion protein purification kit (catalog no. 78100; Thermo Scientific). Briefly, a cell pellet was harvested from 250 ml of IPTG-induced BL21 cells harboring pET21a-brpt1.0 and suspended in 10 ml of B-PER reagent for 30 min of shaking incubation at room temperature. Soluble proteins were separated from insoluble proteins by centrifugation at 27,000 × g for 15 min and applied to a nickel-chelated column. Nonspecific binding proteins were washed away by wash buffers. The peptide 6×His-tagged Brpt1.0 was eluted in elution buffer and further dialyzed using Slide-A-Lyzer dialysis cassettes (Thermo) to remove excess imidazole. The purified Brpt1.0 protein was analyzed by SDS-PAGE, and the concentration was measured using the NanoDrop 2000 (Thermo) at 280 nm. The purified Brpt1.0 protein was suspended in endotoxin-free phosphate-buffered saline (PBS) and stored at −80°C until used.

**Brpt1.0 immunization.** The animal studies were approved by the animal care and use committee of the University of Washington (protocol 4192-01) and performed under specific-pathogen-free (SPF) conditions. Six-week-old female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME), housed in the university animal facility, and supplied with sterile food and water.

In order for us to examine the immunogenicity of the recombinant protein Brpt1.0, 10 mice were subcutaneously injected with 100 μg purified Brpt1.0 in 50 μl endotoxin-free PBS mixed with an equal volume of complete Freund’s adjuvant for the first dose and with incomplete Freund’s adjuvant for the booster. Ten mice serving as sham-treated controls received 100 μg of EndoGrade ovalbumin protein (Ova) (catalog no. 321000; Bioventor) in 50 μl PBS with an equal volume of Freund’s adjuvants. All the mice were immunized subcutaneously on days 0 and 10, and blood samples were collected before immunization and on days 7, 14, 18, and 20 after the first immunization. Sera were pooled in each group of 10 mice at each time point for the antibody assays.

**S. epidermidis RP62A implant infection.** For our examination whether immunization with Brpt1.0 protein protected against *S. epidermidis* infection, both groups of mice received an *S. epidermidis* RP62A implant infection on day 17 after two immunizations. The experimental *S. epidermidis* implantation model was developed based on previous reports (22–24) and optimized by our group (25, 26). Custom-made round, porous template poly(2-hydroxyethyl methacrylate) [poly(HEMA)] scaffold disks (diameter, 7 mm; thickness, 0.5 mm; pore diameter, −35 μm) were used as the implant substances in this study. The porous templating process provides a polymer scaffold where all the pores and pore interconnections are the same size, with both parameters controlled by the spherical template size. Scaffold disks were soaked individually in 10 ml *S. epidermidis* RP62A cultures in their stationary phase and incubated under shaking conditions (125 rpm) for 6 h at 37°C to enable bacterial colonization and growth within the scaffolds. After incubation, the scaffold disks were rinsed 3 times prior to implantation with PBS to remove any loosely held planktonic bacteria on the surface of the scaffolds. To quantify the number of bacteria loaded within the disks, three scaffold disks incubated with bacteria were randomly selected, placed in a 10-ml tube with 1.5 ml PBS, and sonicated for 5 s three times using a needle ultrasonicator to dislodge the adherent bacteria from the disk. The solution was then serially diluted and placed on TSB agar plates for CFU counting after 18 to 24 h of incubation (26). Our preliminary studies indicated that the appropriate amount (5 × 10^6 CFU) of bacteria accumulated within each disk after 6 h of incubation. Confirmatory CFU counting of implantation disks was also performed to quantify the amount of bacteria loaded within the implantation disks.

The entire implantation process was performed in a laminar flow cabinet, and all the mice received isoflurane anesthesia before surgery. The back of each mouse was shaved and sterilized by 75% ethanol before a 10-mm incision was made by a surgical blade. The scaffold disk pretreated with bacteria was implanted subcutaneously through an incision into the dorsal subcutis 1 cm lateral to the spine, and the incision was closed with two surgical wound clips. After implantation, the mice were observed daily and monitored for weight change for 7 days. Blood samples were collected on days 1 and 3 postimplantation (days 18 and 20 postimmunization, respectively) to determine bacterial burden and antibody responses. All the mice were sacrificed under full anesthesia on day 8 postimplantation, and the implants and surrounding tissue were collected for histological analysis.

**Serum antibody ELISA.** Sera were isolated from blood samples collected on days 0, 7, 14, 18, and 20, and antibodies binding *S. epidermidis* cells and Aap were measured by enzyme-linked immunosorbent assay (ELISA). For the anti-*S. epidermidis* ELISA, overnight cultures of *S. epidermidis* RP62A were harvested and washed with PBS three times before being placed on a 100°C thermal block for 1 h. For the anti-Aap ELISA, Aap was isolated from immobilized *S. epidermidis* RP62A cells and purified with an anion-exchange chromatography column as described previously (21). One hundred microliters of heat-killed bacteria at an optical density at 600 nm (OD_{600}) of 1 or 5 μg/ml purified Aap in coating buffer (KPL) was coated onto the wells of a 96-well plate (Costar 3370; BD) for 18 h of incubation at 4°C. After blocking each well with 1% bovine serum albumin (BSA) in PBS, 5-fold serial-diluted serum samples (starting at 1:50) were added to individual wells coated with either *S. epidermidis* cells or Aap at 1 h at 37°C. The detection of any bound immunoglobulin was achieved using 0.1 μg/ml of a secondary horseradish peroxidase (HRP)-conjugated anti-mouse IgG (BioLegend) for 30 min at 37°C. To determine the IgG isotypes, 0.1 μg/ml of anti-mouse IgG1-HRP or IgG2c-HRP conjugates (Abcam) were used instead. The HRP was quantified by 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (BD), with the optical density measured on an ELISA plate reader at 450 nm with a 570-nm correction. Antibody titers were defined as the highest dilution that gave an OD_{450} value greater than the mean plus 3 standard deviations (SD) of preimmune serum. The results were determined using three repeat measurements, and at least two independent experiments were performed for each sample.

**Biofilm inhibition assay.** Biofilms were formed using a semiquantitative microtiter plate assay as previously described (12). Briefly, *S. epidermidis* RP62A cells were harvested from an overnight planktonic culture in TSB and suspended in fresh 2× TSB to an OD_{600} of (1 × 10^7 to 2 × 10^7/ml). For the protein biofilm inhibition assay, 5-fold serial-diluted protein Brpt1.0 (starting at 10 μg/ml) in PBS supplemented with 1 mM ZnCl₂ was added into the wells of a flat-bottom tissue culture-treated 96-well plate (Costar 3595; BD) and mixed with an equal volume of *S. epidermidis* RP62A bacterial suspension. Bovine serum albumin (BSA) served as a negative control to exclude nonspecific blocking of cell attachment. For the serum biofilm inhibition assay, mouse sera were diluted 1:1, 1:100, 1:1,200, and 1:400 in PBS buffer and mixed with equal volumes of bacterial suspension in the individual wells of a 96-well plate. The plate was placed at 4°C for 2 h before incubation at 37°C for 24 h without shaking. A bacterial suspension with preimmune serum was used as a control. After 24 h of culture, cell suspensions were removed, and any biofilm cells were stained with 100 μl of 1% (wt/vol) crystal violet (Sigma) for 10 min at room temperature. The stained biofilms were rinsed with distilled water and air-dried for 30 min before dissolving in 100 μl of 30% (vol/vol) acetic acid. The OD values were read with a UV/visible Safire2 plate reader (Tecan, Manfolden, Switzerland) at 595 nm. The percent inhibition of biofilm formation was determined using the formulas (1 − OD_{sample} with protein/OD_{sample} without protein) × 100% in the protein biofilm inhibition assay and (1 − OD_{sample} serum sample/OD_{sample} naive serum) × 100% in the serum biofilm inhibition assay. Each sample was processed in triplicate and examined in two independent experiments.

**Histological assays.** All mice were euthanized by CO₂ asphyxiation on day 8 postimplantation, and the poly(HEMA) implants together with the
surrounding tissue were excised for histological analysis. All explanted samples were fixed in zinc fixative overnight and embedded in paraffin wax. For each implant, 6-μm sections were cut and mounted onto microscopic slides for three histological stain assays: (i) inflammatory cell infiltration and fibrosis in the tissue samples were examined using Masson’s trichrome stain, which stains cytoplasm red, collagen blue, and nuclei black, (ii) bacterial colonization in implanted scaffold disks and peri-implant tissues was examined by the Brown-Brenn Gram stain, which stains Gram-positive bacteria purple and Gram-negative bacteria and tissue cell nuclei red, and (iii) macrophage presence was examined using an F4/80 immunofluorescence stain as reported previously (27). In the Gram stain assay, the number of Gram-positive cocci present in the implanted scaffold disks and surrounding tissue sections was estimated by counting the cocci captured in five randomly selected 320-μm by 240-μm fields and converting the mean number into bacterial numbers in 10-mm² units, based on previously published methods (24, 27–29) with some modifications. Semi-quantitative bacterial colony counting was performed by three independent researchers blinded to the samples’ identities with at least five independent random images/fields per section on two sections per animal. In the macrophage F4/80 immunofluorescence stain assay, a rat anti-mouse F4/80 antibody (BM8) (1:66 dilution) (Bioscience) and a second Alexa Fluor 594-labeled donkey antibody anti-rat IgG (1:200 dilution) (catalog no. A-21209; Invitrogen) were used to mark pan-macrophages in the scaffold and peri-implant tissues. Images were acquired on a Nikon E800 upright microscope in bright field or epifluorescence and archived using Metamorph software.

Blood culture. To examine the presence of bacteria in the bloodstream after implant infection, 25 μl of blood samples collected on days 1 and 3 postimplantation were serially diluted in TSB medium and immediately cultured on TSB agar plates overnight at 30°C. Blood bacterial cultures from all the samples were negative.

Statistical analysis. The data are presented as means ± standard deviations (SD) and subjected to statistical analysis by applying Student’s t test; a P value of <0.05 was considered statistically significant.

RESULTS

Brpt1.0 inhibits S. epidermidis biofilm formation. The recombinant protein Brpt1.0 was expressed in E. coli in fusion with an N-terminal T7 tag and a C-terminal His tag (Fig. 1A) and purified by His tag metal-chelate affinity chromatography (Fig. 1B). SDS-PAGE analysis of the soluble fraction and insoluble inclusion bodies of IPTG-induced cells indicated that Brpt1.0 was expressed in soluble form (data not shown). A total of 2.5 mg purified protein was harvested in 250 ml of cell culture.

To determine whether the recombinant Brpt1.0 is capable of inhibiting biofilm formation, serial-diluted fusion-expressed Brpt1.0 was added to the S. epidermidis RP62A culture for 24 h of incubation. While BSA as a negative control had no influence on S. epidermidis RP62A biofilm formation, we observed significant dose-dependent biofilm inhibition by recombinant Brpt1.0: 10 μg/ml, 2 μg/ml, and 0.4 μg/ml of Brpt1.0 abolished S. epidermidis RP62A biofilm formation by 33%, 24%, and 10%, respectively (Fig. 1C), indicating that the recombinant single B-repeat domain of Aap has bioactivity in mediating intercellular adhesion and biofilm formation.

Brpt1.0 induced strong antibody responses in mice. To examine the immunogenicity of recombinant protein Brpt1.0, C57BL/6J mice received two subcutaneous immunizations with 100 μg Brpt1.0 mixed with complete Freund’s adjuvants (CFA) on day 0 and incomplete Freund’s adjuvants (IFA) on day 10, followed by an implant challenge with 5 × 10⁶ CFU of S. epidermidis RP62A contained within porous poly(HEMA) scaffold disks on day 17. Mouse blood was withdrawn before and after each immunization (days 0, 7, and 14) and after the implant challenge (days 18 and 20). ELISAs were performed on the sera to measure the IgG antibody binding to the heat-killed S. epidermidis RP62A cells and the purified Aap. IgG isotypes IgG1 and IgG2c in the sera of immunized mice were also analyzed by ELISA.

First, individual serum samples collected on day 14 were analyzed to measure the vaccine-immunized IgG antibodies after two immunizations. Significantly higher IgG antibody responses specific to S. epidermidis cells were observed in Brpt1.0-immunized mice than in Ova-immunized mice (Fig. 2A). ELISAs were performed on the sera to measure the IgG antibody binding to the heat-killed S. epidermidis RP62A cells and the purified Aap. IgG isotypes IgG1 and IgG2c in the sera of immunized mice were also analyzed by ELISA.

FIG 2 The anti-S. epidermidis (anti-S.E.) antibody responses were determined by ELISA. Each mouse was immunized s.c. with either Brpt1.0 or Ova on days 0 and 10 and implanted with a porous poly(HEMA) scaffold preseeded with 5 × 10⁶ CFU of S. epidermidis RP62A on day 17. Murine sera were collected on days 0, 7, 14, 18, and 20. (A) Total IgG antibody responses in individual sera collected on day 14 (after two immunizations) were analyzed by ELISA binding to heat-killed S. epidermidis cells. (B) Kinetics of anti-S.E. IgG antibody development in pooled sera collected on days 0, 7, 14, 18, and 20. (C) Anti-S.E. isotype IgG1 and IgG2c antibody responses of sera collected on day 14. Error bars indicate standard deviations (SD). ***, P < 0.01, and ***, P < 0.001, indicate statistical significances compared with the Ova control group. All samples were tested in triplicate, and ELISAs were repeated at least twice.
Brpt1.0-immunized mice developed high levels of antibodies, while sera from the Ova-immunized mice remained negative for either S. epidermidis cells or Aap; there were no significant individual differences between the mice in either group (Fig. 2A; see also Fig. S1A). Next, we examined the kinetics of antibody responses using pooled serum samples collected at all time points. We observed that Brpt1.0-immunized mice exhibited weak antibody responses on day 7 (with no statistical differences versus Ova-immunized mice) but developed significantly robust IgG antibody responses specific to S. epidermidis cells on day 14 and later (Fig. 2B), suggesting that two subcutaneous immunizations with recombinant protein Brpt1.0 are necessary to induce strong antibody responses against S. epidermidis. We also observed further augmentation of anti-S. epidermidis antibodies in the sera of Brpt1.0-immunized mice on days 18 and 20 and a mild anti-S. epidermidis antibody response in Ova-immunized mice on day 20 (Fig. 2B), most likely due to the S. epidermidis RP62A-filled implant challenge in all the mice on day 17. Similar to the anti-S. epidermidis responses, antibody responses against the protein Aap were negative in the sera of mice after the initial immunization with Brpt1.0, but a significant increase was exhibited after the boost immunization and the implant infection, while Ova-immunized mice remained negative for Aap until after the implant challenge on day 17 (see Fig. S1B). To reveal the type of immune response, we compared the Th2-associated IgG1 and Th1-associated IgG2c levels in the sera of immunized mice on day 14. While all the mice lost weight on day 1 and started to recover on day 3, Brpt1.0-immunized mice lost relatively less weight and started gaining weight at a significantly faster pace than the Ova-immunized mice, starting at day 4 (Fig. 4A). No significant inflammatory reactions were observed at the implantation sites of Brpt1.0-immunized mice, while the Ova-immunized mice developed focal subcutaneous abscesses on day 8 after S. epidermidis RP62A implant infection (Fig. 4B). Consistent with an inflammatory skin reaction, Masson’s trichrome staining of biopsy samples collected on day 8 postimplantation confirmed the presence of severe inflammatory cell infiltration and accumulation at the scaffold disk-tissue interface and inside the scaffold in Ova-immunized mice, while a much milder inflammatory reaction was seen in Brpt1.0-immunized mice (Fig. 4C). The weight changes and inflammatory reactions of the mice postchallenge suggest that Brpt1.0-induced protective immunity in C57BL/6J mice against S. epidermidis RP62A implant infection.

We further investigated bacterial colonization and macrophage presence in the implanted scaffolds and surrounding tissues by Gram stain and macrophage F4/80 stain assays. Although we did not detect any bacteria in the blood of the mice postinfection, we observed bacteria in the scaffolds and in the peri-implant tissues through Gram staining (Fig. 5A to C). Semiquantitative bacterial colony counts indicated a significantly smaller amount of bacteria in the implanted scaffold disk and the surrounding tissue sections from Brpt1.0-immunized mice than in those from the Ova-immunized mice (Table 1). The sample sections were also stained with the pan-macrophage marker monoclonal antibody F4/80. While a large number of macrophages were observed in the scaffold disks and in the surrounding tissue after implantation of contaminated scaffold disks (Fig. 6), fewer macrophages were present in scaffold disks implanted in Brpt1.0-immunized mice than in those implanted in Ova-immunized mice (Fig. 6B). Thectually implanted with a porous poly(HEMA) scaffold disk seeded with $5 \times 10^6$ CFU of S. epidermidis RP62A. While all mice in the Brpt1.0- and Ova-immunized groups survived after the infection challenge, the mice immunized with Brpt1.0 exhibited significant resistance to implant infection by S. epidermidis.

We measured the daily weight changes of mice postimplantation and normalized them to each animal’s initial weight on day 0. While all the mice lost weight on day 1 and started to recover on day 3, Brpt1.0-immunized mice lost relatively less weight and started gaining weight at a significantly faster pace than the Ova-immunized mice, starting at day 4 (Fig. 4A). No significant inflammatory reactions were observed at the implantation sites of Brpt1.0-immunized mice, while the Ova-immunized mice developed focal subcutaneous abscesses on day 8 after S. epidermidis RP62A implant infection (Fig. 4B). Consistent with an inflammatory skin reaction, Masson’s trichrome staining of biopsy samples collected on day 8 postimplantation confirmed the presence of severe inflammatory cell infiltration and accumulation at the scaffold disk-tissue interface and inside the scaffold in Ova-immunized mice, while a much milder inflammatory reaction was seen in Brpt1.0-immunized mice (Fig. 4C). The weight changes and inflammatory reactions of the mice postchallenge suggest that Brpt1.0-induced protective immunity in C57BL/6J mice against S. epidermidis RP62A implant infection.

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DISCUSSION

Previous studies from our lab and those of others have established that antiserum against Aap prevents *S. epidermidis* biofilm formation *in vitro* (11, 14, 15, 21), indicating the potential of Aap as a biofilm vaccine candidate. Recent studies with direct X-ray crystallography further demonstrated that multiple tandem B-repeats in the B region of Aap form twisted-rope-like structures between bacterial cells through Zn$^{2+}$-mediated homodimerization and mediate staphylococcal biofilm formation (12, 16). Therefore, in this study, we focused on a single B-repeat of Aap (Brpt1.0) that consists of a 78-aa G5 domain and a 50-aa spacer segment and investigated its immunogenicity and protective efficacy against staphylococcal biofilm infection in an experimental model of biomaterial-associated infection in mice.

Although it was suggested that a single C-terminal B-repeat might not be as stable as a single B-repeat followed by the half repeat (Brpt1.5) (12, 16), we did not observe any instability of Brpt1.0 during the expression and purification processes. The Brpt1.0 used in this study was the N-terminal B-repeat, which carries 18 different amino acids, compared to the C-terminal B-repeat used in other reports (12, 16). The fusion expression of Brpt1.0 with an N-terminal T7 tag and C-terminal His tag probably also contributed to the protein’s stability. Furthermore, our study demonstrated that the *E. coli*-expressed fusion protein Brpt1.0 was capable of inhibiting *S. epidermidis* RP62A biofilm formation *in vitro*. These results support previous observations of biofilm inhibition using a recombinant B-region fused with an N-terminal His tag (15) or a single C-terminal B-repeat followed by the half repeat fused with maltose-binding protein (MBP-Brpt1.5) (12), revealing that Aap mediates intercellular adhesion in *S. epidermidis* biofilms through the B-repeat region.

The immunogenicity assay using Brpt1.0 coadministered with Freund’s adjuvant in C57BL/6J mice demonstrated that a single B-repeat of *S. epidermidis* Aap is capable of inducing high levels of antibodies against *S. epidermidis* cells. In addition, the kinetics of vaccine-induced antibody development indicated that a booster immunization with the Brpt1.0 vaccine was necessary following the initial subcutaneous immunization in order to induce strong antibody responses against *S. epidermidis* infections in mice. While IgG1 and IgG2a antibodies are widely used as indirect measures of T helper 1 (Th1)- and Th2-type immune responses, certain strains of mice, including C57BL/6, C57BL/10, SJL, and NOD, lack the Igh-1a allele that codes for IgG2a but instead express IgG2c from the Igh-1b allele (30–32). Hence, in this study, we compared the specific IgG1 and IgG2c antibody titers in the sera of immunized mice. We observed significantly higher IgG1 levels than IgG2c levels for both *S. epidermidis* and Aap, indicating that immunization with recombinant Brpt1.0 coadministered with Freund’s adjuvant promoted a Th2-dominant immune response in C57BL/6 mice.

Our study also indicated that Brpt1.0-induced antisera were capable of neutralizing *S. epidermidis* RP62A and inhibiting biofilm formation *in vitro*. Sera from Brpt1.0-immunized mice exhibited a significantly greater ability to inhibit biofilm formation in a dose-dependent manner than those from Ova-immunized mice. It was not surprising that the elevated IgG levels in the Ova-immunized mice displayed some inhibition of *S. epidermidis* biofilms through the B-repeat region. However, our study demonstrated that antiserum biofilm inhibition assay on various doses (0.005 to 10 mg/mouse) administered intraperitoneally simultaneously with a local bacterial challenge significantly increased mouse survival in a dose-dependent manner, which is consistent with our observation of the antiserum biofilm inhibition assay on *E. coli in vitro*. Our study indicated that while there are other elements in sera that interfere with biofilm formation, Brpt1.0-specific antibodies exhibit an enhanced ability to inhibit *S. epidermidis* RP62A biofilm formation *in vitro*, indicating the potential of Brpt1.0 as a biofilm vaccine against *Staphylococcus* biofilm infection in vivo.
While it has been reported that MAbs and antiserum against Aap prevent *S. epidermidis* biofilm formation *in vitro* (11, 14, 15, 21), a mouse biomaterial-associated infection model indicated that passive immunization with MAbs against Aap increased rather than reduced *S. epidermidis* adherence to the implants *in vivo* (37). Also note that in a recent *in vitro* study on three MAbs generated against Aap, Brpt1.5 showed that one MAb (18B6) inhibited *S. epidermidis* RP62A biofilm accumulation, while two other MAbs (20B9 and 25C11) enhanced biofilm formation *in vitro* (38), indicating that different epitopes of a vaccine target can induce contrasting effects and that passive immunization with MAbs against a single Aap epitope may not block *S. epidermidis* biofilm accumulation. In our study, we demonstrated, for the first time, that a preventive immunization with recombinant Brpt1.0 coadministered with Freund’s adjuvant can protect mice against *S. epidermidis* RP62A infection in an experimental biomaterial-associated infection model.

Given that *S. epidermidis* does not produce aggressive virulence factors in commensal lifestyle until it switches to biofilm-forming cells upon medial device colonization (4), our preliminary studies (data not shown) indicated that direct injection with up to $10^7$ cells/ml *S. epidermidis* RP62A through either the subcutaneous (s.c.) or intravenous (i.v.) routes did not cause any adverse reactions in C57BL/6 mice and did not lead to death. Therefore, an experimental biomaterial-associated infection mouse model was required to evaluate the protective efficacy of the Brpt1.0 vaccine.

### TABLE 1 Bacterial cluster counts in biopsy samples

| Sample type | Cluster count (per 10 mm²) in mice immunized with: |
|-------------|--------------------------------------------------|
|             | Brpt1.0 | OVA | *P* |
| Tissue      | 1,025 ± 788 | 5,900 ± 1,943 | 0.0008* |
| Scaffold    | 3,300 ± 1,826 | 13,450 ± 5,113 | 0.0031** |

* Data are expressed as means ± standard deviations.

* *P* values show statistical significances between 2 groups: *P* < 0.001; **P* < 0.01.

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FIG 5 Gram-positive bacterial colonization observations in biopsy samples from C57BL/6 mice on day 8 postimplant challenge with $5 \times 10^6$ CFU of *S. epidermidis* RP62A. (A) Representative Gram stain of peri-implant tissue (T) and implanted scaffold (S) sections. (B) Magnification of the peri-implant tissue sections in panel A. (C) Magnification of the implanted scaffold sections in panel A. Arrows point to examples of bacterial clusters.
In this study, we utilized porous (35-μm pore size) poly(2-hydroxyethyl methacrylate) [poly(HEMA)] scaffold disks as bacterium-loaded constructs containing 5 × 10^6 CFU of *S. epidermidis* RP62A. Weight changes, observations of inflammation, and histological assay results after a device-based *S. epidermidis* infection challenge indicated that the mice immunized with Brpt1.0 exhibited significantly higher resistance to *S. epidermidis* RP62A implant infection than the Ova-immunized mice. The histology of the infected scaffolds from Ova-immunized mice revealed severe inflammatory responses and a high residual population of macrophages coincident with high numbers of residual bacteria versus the Brpt1.0-immunized mice, which had presumably mounted a successful innate response to the infected scaffold disk. In future studies, a time-lapse histological study from day 1 postimplantation to day 8 would be necessary to substantiate this suggested enhanced innate response.

In conclusion, our study emphasizes the immunogenicity and protective efficacy of Brpt1.0 in an experimental model of biomaterial-associated infection in mice and demonstrated, for the first time, that immunizations with a single B-repeat of *S. epidermidis* Aap can induce protective immune responses against implant infection with *S. epidermidis* RP62A in C57BL/6 mice. These findings lay the foundation for the future development of new-generation vaccines (such as nucleic acid vaccines) against biomaterial-associated infections by any colonizing bacteria.

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**FIG 6** Macrophage observations of biopsy samples from C57BL/6 mice on day 8 postimplantation. Representative fluorescence images of macrophage F4/80 staining of biopsy samples from mice in each group (A) and magnification of the implanted scaffold sections (S) in panel A (B) indicate fewer macrophages present in scaffolds implanted in Brpt1.0-immunized mice than in Ova-immunized mice.
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