Attenuation of Staphylococcus aureus–Induced Bacteremia by Human Mini-Antibodies Targeting the Complement Inhibitory Protein Efb

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Staphylococcus aureus can cause a broad range of potentially fatal inflammatory complications (e.g., sepsis and endocarditis). Its emerging antibiotic resistance and formidable immune evasion arsenal have emphasized the need for more effective antimicrobial approaches. Complement is an innate immune sensor that rapidly responds to bacterial infection eliciting C3-mediated opsonophagocytic and immunomodulatory responses. Extracellular fibrinogen-binding protein (Efb) is a key immune evasion protein of S. aureus that intercepts complement at the level of C3. To date, Efb has not been explored as a target for mAb-based antimicrobial therapeutics. In this study, we have isolated donor-derived anti-Efb IgGs that attenuate S. aureus survival through increased neutrophil killing. A phage library screen yielded mini-Abs that selectively inhibit the interaction of Efb with C3 partly by disrupting contacts essential for complex formation. Surface plasmon resonance–based kinetic analysis enabled the selection of mini-Abs with favorable Efb-binding profiles as therapeutic leads. Mini-Ab–mediated blockade of Efb attenuated S. aureus survival in a whole blood model of bacteremia. This neutralizing effect was associated with enhanced neutrophil-mediated killing of S. aureus, increased C5a release, and modulation of IL-6 secretion. Finally, these mini-Abs afforded protection from S. aureus–induced bacteremia in a murine renal abscess model, attenuating bacterial inflammation in kidneys. Overall, these findings are anticipated to pave the way toward novel Ab-based therapeutics for S. aureus–related diseases. The Journal of Immunology, 2015, 195: 000–000.

Staphylococcus aureus is a human pathogen that has an increasingly negative impact on human health (1). It is a leading cause of hospital and community-acquired infections ranging from skin or soft tissue abscesses to severe necrotizing infections with a highly invasive phenotype (2).
S. aureus targets that are instrumental in promoting the pathogen’s virulence (7, 12, 13).

Complement is a crucial innate immune sentinel that protects from bacterial infection by triggering a cascade of protein–protein interactions that lead to C3-mediated pathogen opsonophagocytosis (14). S. aureus has evolved several strategies to subvert complement by secreting molecules that selectively inhibit distinct components and activation pathways (15–17). Among an array of secreted proteins, S. aureus encodes extracellular fibrinogen-binding protein (Efb), a key immune-evasion protein that selectively targets the complement component C3 (18). Efb is a 16-kDa protein that consists of two structurally and functionally distinct domains: an N-terminal domain (1–64 aa), which binds to fibrinogen (Fg) (19), and the C terminus domain (Efb-C; 65–165 aa), which binds to C3 and its fragments C3b and C3d (18). Efb blocks complement activation by allosterically inhibiting formation of C3b-containing C3 and C5 convertases (18, 20, 21). In vivo studies using mutant S. aureus strains lacking Efb have revealed its virulence-promoting role in S. aureus pathogenesis (22–24).

Exploiting the potent complement-modulatory activity of Efb and its crucial role in promoting S. aureus’ infectivity, we have developed and characterized recombinant human mini-Abs that neutralize the function of Efb both in vitro and in vivo by blocking its interaction with complement C3. These targeted antimicrobial agents potently attenuate the survival of S. aureus in models of bacteremia and show promise for further development as Ab-based vaccine candidates for passive immunization.

Materials and Methods

Proteins/reagents

Mini-Abs against Efb-C were generated by screening a HuCal Ab library (Bio-Rad ABD Serotec, Puchheim, Germany) as described previously (25). A mini-Ab consists of a dimeric Fab fragment linked together by an oligomerization domain flanked by two epitope tags (25). C3 and C3d were purified based on previous protocols (18, 26, 27). Recombinant Efb, Efb-C, Efb-C-RENE, Ehp, and SCIN-A proteins were expressed and purified as described previously (28). S. aureus strain Newman was purchased from American Type Culture Collection (25004).

Human plasma samples

Routine clinical blood specimens along with clinical information were obtained from patients admitted at the General Hospital “Evangelismos” under an approved human subject protocol. Patients were characterized as S. aureus–positive (i.e., presenting infections of the blood, lung, or skin) based on microbiology laboratory reports. Blood was obtained from healthy individuals who had previously been identified as S. aureus–negative (i.e., no active infection at the time of blood sampling).

Whole blood model of bacteremia

Experiments with blood from healthy donors were performed as described previously (29). The whole blood model was applied in two distinct formats: 1) the “endogenous” format—briefly, S. aureus cells (1X10⁶ CFU/ml) were incubated with 50% whole blood in the presence of mini-Abs for 4 h at 37°C; and 2) the “exogenous” format—S. aureus cells (2.5X10⁶ CFU/ml) were incubated with 40% whole blood in the presence of 1.5 μmol recombinant Efb-C for 2 h at 37°C after the addition of mini-Abs. After incubation, a small volume of the samples was plated for calculation of S. aureus survival rate. The remaining volume of the samples was centrifuged for plasma collection.

Neutrophil killing assay

To assess neutrophil killing, S. aureus cells (Newman strain) at 5X10⁶ CFU/ml were preincubated for 15 min with 10% human serum, followed by incubation with 1X10⁶ neutrophils for 30 min at 37°C. A total of 50 nmol mini-Abs or human donor-derived IgGs were added to S. aureus suspensions during the preincubation step, in the presence of 50 nmol Efb-C. S. aureus killing was analyzed on tryptic soy broth (TSB) agar plates. The percentage of cell killing was calculated based on the formula T₀ – Tₜ/T₀ × 100.

Isolation of anti–Efb-C IgG from human plasma

Human plasma verified by ELISA for the presence of anti–Efb-C IgG (see below) was applied to a CNBr-activated Sepharose-4B column (GE Healthcare) in which Efb-C had been immobilized. After extensive washing with PBS, IgG bound to Efb-C were eluted with 0.1 mol glycine (pH 2.5). Eluted fractions were immediately neutralized by addition of Tris-HCl (pH 8) and dialyzed overnight against PBS at 4°C. As a negative control for all subsequent studies, plasma was passed through the same column, and the unbound material was collected. Next, IgG were isolated from the unbound fraction, and lack of anti–Efb-C IgG was confirmed by ELISA.

Surface plasmon resonance

Selectivity profile of mini-Abs. The interaction between the mini-Abs and S. aureus proteins was measured using a Biacore 3000 (GE Healthcare) at 25°C. HBS-EP (0.01 mol HEPES [pH 7.4], 150 mmol NaCl, 3 mmol EDTA, and 0.005% (v/v) Tween-20) was used as the mobile phase throughout the experiment. Anti-human Fab Abs (Fab binder Kit; GE Healthcare) were immobilized at a density of 10,000 resonance units (RU) on a CMS sensor chip according to the manufacturer’s instructions. Mini-Abs were captured on individual flow cells of the Fab-binding sensor chip at a density of <600 RU. S. aureus proteins were diluted to a concentration of 100 nmol in HBS-EP and injected for 1 min at a 20 μl/min flow rate with a dissociation phase of 2 min. At the end of each screening cycle, the mini-Abs were stripped off the surface by injecting glycine buffer (pH 2.1) twice for 1 min each to allow for capturing of a new mini-Ab set. Signals from an empty Fab-binder flow cell and an ensemble of buffer blank injections were used to perform double referencing of samples responses. Data processing and evaluation was performed using Scrubber (v2.0; BioLogic Software, Campbell, Australia).

Kinetic analysis of the mini-Ab/Efb-C interaction. For the kinetic studies, mini-Abs were captured at a density of 500 RU on individual flow cells as described above. A single cycle kinetic titration model was employed (30), in which five concentrations of Efb-C spanning a 5-fold dilution series (3.3–270 nmol) were injected for 2 min each without intermittent regeneration. At the end of each cycle, the dissociation was observed for 5 min, and mini-Abs were stripped off the surface. Three buffer blanks were included in each analysis, and their average signals were subtracted from the processed sample responses (double referencing). Each data set was fitted to a Langmuir 1:1 interaction model (single-cycle method kindly provided by GE Healthcare) and the equilibrium dissociation constant (KD) was calculated.

Immunoblotting

S. aureus strains Newman, ∆Efb (kindly provided by Dr. Suzan Rooijakkers, University Medical Center Utrecht, the Netherlands), and ∆Efp were grown in TSB medium for 20 h at 37°C. Following cell sedimentation, culture supernatants were concentrated with 13% TCA. Samples were run on an SDS-PAGE gel and transferred onto a nitrocellulose membrane. After blocking of the membrane with 5% milk/PBS/Tween-20 (0.05%) for 1 h, the polyclonal Ab anti–Efb-C or the mini-Abs were added at final dilution 1/100 or 1 μg/ml, respectively, and incubated for 1 h. Next, the membrane was washed twice with PBS/Tween-20 followed by incubation with the anti-rabbit-HRP (Bio-Rad) or anti-mouse-HRP at 1/2000 dilution. Immunoreactive bands were visualized by chemiluminescence (ECL kit; GE Healthcare) according to the manufacturer’s instructions on a Fujifilm LAS-4000 luminescent image analyzer (Fujifilm Manufacturing USA, Greenwood, SC).

ELISAs

Anti-Eb IgG detection in human plasma. For the detection of anti-Eb IgG in plasma from S. aureus–positive and –negative human donors, 96-well ELISA plates were coated with 2 μg/ml Eb-C overnight at 4°C. After blocking the wells with 1% BSA/PBS for 1 h at room temperature, plasma samples were serially diluted and incubated for 30 min. Following washing of the wells with PBS/Tween-20, the polyclonal Ab anti-human IgG-HRP was added at 1/10,000. Samples were incubated for 30 min, and after extensive washing, immune complexes were detected at 405 nm.

Competitive ELISA for measuring Ab-mediated inhibition of binding of C3 fragments to Eb-C

Inhibition by donor-derived IgG. ELISA plates were coated with 5 μg/ml Eb-C as above. After blocking with 5% BSA/PBS/Tween-20 for 1 h at room temperature, donor IgGs were serially diluted starting from a concentration of 10 μg/ml. Following incubation for 45 min, 1 μg/ml C3 was
added to the wells, and the mixture of the proteins was further incubated for 30 min. Following washing, the anti-C3 (clone 755; Hycult Biotech) was added at 2 μg/ml for 30 min. Following the addition of the anti-mouse IgG-HRP (1/2000), the plate was washed, and OD was measured at 405 nm. The results were expressed as percentage of inhibition of Efb-C/C3 binding, using as negative control (0% inhibition) OD values obtained from samples without donor-derived IgGs.

Inhibition by mini-Abs. ELISA plates were coated and blocked as described above. Mini-Abs were serially diluted and incubated for 45 min followed by addition of 1 μg/ml C3/C3b/C3d and further incubation for 30 min. Following washing, the polyclonal anti-C3 (clone 755; Hycult Biotech), anti-C3b (clone 13/15; Hycult Biotech), and H8S (anti-human C3d) (31) were added at 2 μg/ml for 30 min. Following the addition of the anti-mouse IgG-HRP (1/2000), the plate was washed, and immune complexes were detected as above. The results were expressed as percentage of inhibition of Efb-C/C3/C3b/C3d binding, using as negative control (0% inhibition) values obtained from samples without mini-Abs.

Efb detection in whole blood. Lepirudin-anticoagulated whole blood was incubated with S. aureus, Newmann strain (1 × 10⁶ CFU/ml), and samples were collected at various time intervals (0–12 h). Plasma was recovered and analyzed for the presence of Efb according to the following ELISA scheme (32): ELISA plates were coated with 1 μg/ml fibrinogen (Sigma-Aldrich) as above. After blocking with 2% BSA/PBS, plasma samples were serially diluted and incubated for 1 h at 37°C. Following washing, a polyclonal anti-Efb-B Ab was added at 1/100, followed by an anti-rabbit IgG-HRP at 1/2000 for 30 min. After washing, immune complexes were detected at 405 nm. The results were expressed in arbitrary units corresponding to the levels of Efb.

ELISA for C5a detection. ELISA plates were coated with the anti-human C5a mAb (R&D Systems, Minneapolis, MN) as described above. After blocking, plasma samples were serially diluted and incubated for 30 min. Following washing, a polyclonal anti-human C5a (CompTech, Tyler, TX) was added at a dilution of 1/2000 for 30 min. Following washing, anti-rabbit HRP-IgG was added at 1/2000 for 30 min, and immune complexes were measured at 405 nm. A standard curve of recombinant human C5a was used to quantify C5a levels in plasma (in nanograms per milliliter).

ELISA for IL-6 detection. The presence of IL-6 in human plasma was quantified by ELISA for IL-6 detection. ELISA plates were coated with the anti-human IL-6 mAb (R&D Systems) at a dilution of 1/2000 for 30 min. Following washing, a polyclonal anti–IL-6 Ab was added at 1/100, followed by biotinylated anti-rabbit IgG (1/2000) and horseradish peroxidase-streptavidin (1/2000). OD was measured at 405 nm. IL-6 concentrations were determined from standard curves prepared from recombinant human IL-6.

Results

Human donors possess neutralizing anti-Efb IgGs that attenuate bacterial survival

To assess the Ab response against Efb and compare the IgG profiles of healthy donors (S. aureus–negative) with those of individuals presenting with S. aureus infections of the skin, lung, etc. (S. aureus–positive), we initially screened by ELISA a large number of serum samples (n = 104/group) for the presence of anti-Efb IgG. This Ab profiling identified higher titers of anti-Efb IgG in S. aureus–negative donors as compared with donors positive for S. aureus infections (p < 0.05) (Fig. 1A). Prompted by this observation, we sought to determine whether such donor-derived anti-Efb Abs would be capable of blocking the complement modulatory function of Efb, which has previously been assigned to the C terminus region of the molecule (Efb-C) (18).

To this end, we set up a competitive ELISA that would allow us to assess the effect of these Abs on the interaction of Efb-C with C3 (Fig. 1B). Our ELISA studies revealed a panel of affinity-purified anti-Efb human IgG that blocked the Efb-C/C3 interaction, reaching ~100% inhibition in most cases (Fig. 1C). Strikingly, these blocking Abs had been isolated from donors that were classified as S. aureus–negative (N3, N4, and N5), whereas Abs isolated from S. aureus–positive donors did not show any blocking effect (P1–P5).

Compartment activation leads to enhanced microbial killing, in part via recruitment and activation of potent phagocytic cells, such as neutrophils (33). In this respect, we next asked whether these blocking Abs could potentiate the neutrophil-mediated killing of S. aureus through their inhibitory effect on complement. Although recombinant Efb-C significantly reduced S. aureus cell killing (p < 0.05), addition of the blocking Abs N3 and N5 effectively restored S. aureus killing to the levels observed in the control sample lacking Efb-C (Fig. 1D).

Discovery of highly selective Efb-C–targeting mini-Abs through a phage library screen

Our initial observations on the presence of neutralizing anti-Efb Abs in human donors and their capacity to promote bacterial cell killing led us to hypothesize that such Abs might have therapeutic value as highly selective antimicrobial agents. However, due to the limited availability of human donor-derived IgG and the potential adverse effects associated with the use of human plasma products in therapeutic applications (11), we focused our efforts on generating human monoclonal anti–Efb-C Abs that would display similar protective antimicrobial properties.

A human mini-Abr library comprising bivalent human F(ab)₂ was screened against the target protein Efb-C, and the panning resulted in 12 mini-Abs with unique primary amino acid sequences. SDS-PAGE analysis revealed highly pure mini-Abr preparations consisting of two distinct bands at the apparent molecular masses of 34 and 26 kDa under reducing conditions (data not shown).

To test the specificity of the generated mini-Abs for Efb-C, we employed a surface plasmon resonance (SPR)–based approach (see schematic representation, Fig. 2A, right panel). To obtain a comprehensive selectivity profile, we also tested the binding of the mini-Abs to the S. aureus–encoded complement modulatory proteins Ehp (34) and SCIN-A (35), which share 44 and 12% sequence identity with Efb-C, respectively. Most mini-Abs were highly selective for Efb-C, because no binding was recorded for Ehp and SCIN-A (Fig. 2A). The only exception was mini-Ab A2, which exhibited a unique selectivity profile binding both Efb-C and its homologous protein Ehp (34).

All 12 mini-Abs were analyzed for their binding affinity to Efb-C. The binding kinetics of those mini-Abs exhibiting the highest binding scores from our initial SPR-based selectivity analysis are shown in Fig. 2B. We observed that the binding profiles of all mini-Abs generally followed a Langmuir 1:1 interaction model. Deviations observed in the kinetic fits of mini-Ab A1 may have been caused by heterogeneity in the protein preparation or target rebinding effects.
Evaluation of the epitope specificity of the mini-Abs for the recombinant proteins Efb, Efb-C, Ehp, and SCIN-A revealed that mini-Abs A1 and A2 likely recognize linear epitopes on proteins Efb and Efb-C (Fig. 2C). No reactivity was observed with the proteins Ehp and SCIN-A, confirming the high selectivity attested to these Abs by our SPR analysis, with the exception of mini-Ab A2, which yielded faint reactivity with the Ehp protein. Attempts to determine whether the mini-Abs also recognize conformational epitopes on Efb using native electrophoresis failed to produce conclusive results due to the high isoelectric point values of the proteins.

To further discern the epitope specificity of the mini-Abs, culture supernatants from wild-type (wt) Newman and ΔEfb and ΔEhp strains were analyzed by immunoblotting (Fig. 2C). Mini-Abs A1 and A2 recognized epitopes on the native Efb protein, which is secreted by the S. aureus strains Newman and ΔEhp.

**Mini-Abs interfere with the binding of Efb-C to C3 and its activated fragments C3b and C3d by disrupting contacts essential for complex formation**

The generation of highly selective mini-Abs recognizing Efb-C prompted us to investigate whether these Abs can exert...
FIGURE 2. Selectivity and binding profiles of the anti–Efb-C mini-Abs generated from a human F(ab’)2 library screening. (A) Left panel, Relative binding activities of the mini-Abs toward selected complement-modulatory proteins of *S. aureus* as determined by SPR analysis. Right panel, Schematic outline of the SPR-based experimental setup employed to determine the selectivity of the various mini-Abs. (B) Kinetic analysis of the interaction between selected mini-Abs and Efb-C. The processed binding curves (black lines) were fitted to kinetic models (gray lines). The *K*ₐ values corresponding to each mini-Ab are shown in the respective sensogram. Data are representative of three independent experiments. (C) Immunoreactivity of selected mini-Abs with (i) a group of selected *S. aureus* recombinant proteins or (ii) culture supernatants from different *S. aureus* strains (Newman, ΔEfb, and ΔEhp). Immunoblot analysis using a polyclonal antiserum raised against Efb-C was performed in parallel to ensure protein presence and integrity.
neutralizing effects by interfering with the binding of Efb-C to its target protein C3. It has previously been shown that Efb-C binds with differential affinity to all thioester-containing C3 fragments besides native C3 (i.e., C3b and C3d) (18). To test if the mini-Abs block the binding of Efb-C to these C3-derived molecules, we performed a competitive ELISA, as described in Fig. 1B. Mini-Abs A1, A2, A3, and A4 displayed the highest inhibitory effects against all C3 fragments tested (C3, C3b, and C3d) (Fig. 3A–C). Interestingly, the same mini-Abs were also selected for having the highest binding affinities for Efb-C (as shown above). On the contrary, mini-Ab C4, which showed negligible binding to Efb-C, did not significantly inhibit the interaction of Efb-C with all the C3 fragments. Previous studies have shown that Efb-C binds C3d with higher affinity than C3 or C3b (18). Confirming this observation, the mini-Ab’s inhibitory dose required for competing out the binding of Efb-C to C3d was considerably higher when compared with C3 and C3b, which was clearly reflected by the calculated IC50 values in each case (Table I).

As stated above, one of the mini-Abs isolated from the phage library screening (i.e., mini-Ab A2) showed comparable binding to both Efb-C and Ehp proteins. However, when tested in the same assay, mini-Ab A2 did not inhibit the interaction of C3d with Ehp (Supplemental Fig. 1). This result can partly be explained by the fact that Ehp has two distinct binding sites on human C3d (34), whereas mini-Ab A2 is likely to interfere with only one of these sites on C3d.

Guided by structural insights of the C3d/Efb-C complex that have revealed the crucial role of specific Efb-C residues in mediating the contact with C3d (18, 36), we next asked whether these mini-Abs recognize epitopes on Efb-C that might contain key structural elements for complex formation. To test this hypothesis, we determined the binding of the inhibitory mini-Abs A1–A4 to the loss-of-function Efb-C mutant proteins Efb-C-(RENE) and Efb-C-(RANA), which have been shown to abolish the binding of Efb-C to C3d (18). As illustrated in Fig. 3D, reduced binding of all mini-Abs to the mutant Efb-C proteins was observed, consistent with our hypothesis about the mini-Ab–mediated interference of complex formation. More specifically, A2 appears to have a particularly high sensitivity for the essential amino acids. In the case of A1, A3, and C1, these effects seem much less pronounced.

Mini-Abs attenuate S. aureus survival in a whole blood model of bacteremia

Our initial studies demonstrated that the mini-Abs block the interaction of Efb-C with C3/C3b, thereby interfering with a key immune evasion mechanism of S. aureus. Based on these findings,
we sought to determine whether these mini-Abs can exert a protective effect by decreasing _S. aureus_ survival in an ex vivo model that closely simulates human bacteremia. To this end, we employed a whole blood model of _S. aureus_ infection (29). Because the mini-Abs specifically target Efb-C, we initially assessed the activity of recombinant Efb-C in this model to validate its key role in pathogen survival (Fig. 4A). Exogenously added Efb-C had a dose-dependent positive effect on _S. aureus_ survival, compared with its inactive counterpart (Efb-C-RENE) (p < 0.05).

Having verified the survival-promoting effect of Efb-C in our system, we then tested the ability of selected inhibitory mini-Abs (A1 and A2) to neutralize this effect, thereby restoring _S. aureus_ growth to basal levels. Both mini-Abs A1 and A2 effectively reversed the Efb-C–dependent effect on _S. aureus_ growth, attenuating bacterial survival in a dose-dependent manner (p < 0.05), (Fig. 4B, right panel).

To translate these findings in a more clinically relevant context, we evaluated the effect of the mini-Abs in a whole blood model of _S. aureus_ bacteremia targeting the native Efb protein that is secreted by _S. aureus_ cells (Fig. 4B, left panel). Despite having the same complement-modulatory activity (18), full-length Efb and Efb-C have not been compared side by side in a whole blood model for their growth-promoting activity on _S. aureus_. A direct comparison of these proteins in our system revealed a similar effect on _S. aureus_ growth (Supplemental Fig. 2). The slightly more pronounced effect of Efb may be explained by the fibrinogen-binding effect that was recently suggested (37).

To establish the time course of Efb secretion in whole blood inoculated with _S. aureus_ (1 × 10⁶ CFU/ml), we first assayed its production at several time points following _S. aureus_ inoculation. As shown in Fig. 4C, _S. aureus_–secreted Efb could be detected by ELISA as early as 2 to 3 h following inoculation and consistently increases for the duration of the experiment (12 h).

Having established that native Efb is secreted in detectable amounts in blood, we then evaluated the neutralizing effect of the mini-Abs A1 and A2 in the whole blood model. Consistent with our findings in the exogenous model, we observed a significant attenuation of _S. aureus_ survival in the presence of these Abs, as compared with control (Fig. 4D).

**Mini-Abs potentiate the neutrophil-mediated killing of _S. aureus_ via complement modulation**

To gain further insight into the mechanism by which the mini-Abs impact _S. aureus_ survival, we determined whether they could restore levels of complement activation counteracting the function of Efb. Although the addition of Efb-C significantly reduced complement activation in _S. aureus_–infected blood, as evidenced by lower C5a levels (Fig. 5A), the blocking mini-Ab A1 could restore C5a generation to a significant extent (25% increase over Efb-C alone).

This observation prompted us to investigate whether this mini-Ab–mediated increase in C5a generation could reflect a greater neutrophil killing activity toward _S. aureus_ cells. Indeed, as shown in Fig. 5B, the blocking mini-Ab A1 abrogated the inhibitory effect of Efb-C on _S. aureus_ killing (28% inhibition), significantly restoring the bactericidal activity of polymorphonuclear neutrophils (PMNs) to approximately basal levels (~70%).

IL-6 is a key proinflammatory cytokine that is elevated during bacterial infection (38). To determine whether mini-Ab–mediated blockade of Efb interferes with cytokine release, thereby attenuating the inflammatory complications of bacteremia, we measured IL-6 levels in _S. aureus_–infected blood treated with blocking mini-Abs. Addition of recombinant Efb-C caused an increase in IL-6 levels (p < 0.05), which was reciprocal to the increase of bacterial burden (data not shown). On the contrary, the blocking mini-Ab A1 caused a significant reduction in IL-6 levels (Fig. 5C), corresponding to the lower bacterial burden observed in the presence of the mini-Ab (see also Fig. 4B).

**Mini-Ab–mediated blockade of Efb confers protection from _S. aureus_–induced inflammation in a murine renal abscess model**

To evaluate the therapeutic efficacy of the most promising blocking mini-Ab in vivo, we employed the _S. aureus_–induced, sublethal renal abscess model (39).

Mice injected with a single i.p. dose of the blocking mini-Ab A1 (1 mg/kg) 2 h prior to staphylococcal infection exhibited a significantly more active behavior and a clinical score that closely resembled that of noninfected control animals (i.e., greater movement, less ruffled fur, and a notable hind limb reflex) throughout the duration of the experiment (until day 10 postinfection). This observation was substantiated by the gross morphology of the kidneys harvested from mini-Ab–injected animals on day 10 (Fig. 6A). Although kidneys from nontreated control ( _S. aureus_–infected) animals displayed large areas of dense inflammatory infiltrates and extensive tissue damage and presence of abscesses with Staphylococcal communities, kidneys from mini-Ab–treated mice showed clear signs of recovery underscored by fewer, less diffuse inflammatory infiltrates and smaller areas of tissue damage, with lower number of detectable Staphylococcal communities (inflammatory score: 6.39 ± 1.95%; p = 0.00014) (Fig. 6C, right panel). Conversely, kidney sections from control animals ( _S. aureus_ infected, no mini-Ab) presented larger areas of inflammation and tissue necrosis (inflammatory score: 25.7 ± 4.93%; p = 0.00014), with greater numbers of staphylococcal communities (Fig. 6C, left panel). These findings were further corroborated by colony enumeration experiments in which kidney homogenates from mini-Ab–treated mice showed a significantly lower bacterial burden (log₁₀ CFU: 3.87 ± 0.44), compared with kidneys from control animals (log₁₀ CFU 5.50 ± 0.55; p = 0.002) (Fig. 6B, Table II).

Prompted by the observation that a single dose of the mini-Ab A1 elicited a prolonged protective effect in _S. aureus_–challenged mice, we assayed the stability of this Ab preparation in mouse serum for an extended time period (10 d). As shown in Supplemental Fig. 4, mini-Ab A1 showed a remarkable stability profile and no signs of proteolytic degradation in mouse serum.

Overall, the significant protection afforded by the Efb-C–targeted mini-Ab in _S. aureus_–challenged mice, as reflected by the

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**Table I. Mini-Abs interfere with the binding of C3 and its activated fragments C3b and C3d to Efb-C**

<table>
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The IC₅₀ values of all mini-Abs are shown with respect to each Efb-C ligand tested.

NA, not applicable.
FIGURE 4. Attenuation of *S. aureus* growth in whole blood by Efb-C–neutralizing mini-Abs. (A) Recombinant Efb-C promotes the growth *S. aureus* (CFU/ml) in whole blood, in a dose-dependent fashion, as compared with inactive control protein. Data represent mean ± SD of three separate experiments. *p < 0.05 with reference to the 0 μmol Efb-C value. (B) Left panel, Schematic overview of the whole blood model of *S. aureus*–induced bacteremia. Our experimental setup included two distinct but also complementary approaches to assess the efficacy of the mini-Abs in blocking *S. aureus* survival: 1) the “exogenous” approach was based on the addition of recombinant Efb to blood mixed with live *S. aureus* cells; whereas 2) the “endogenous” approach relied on the production of native Efb by *S. aureus* cells. Right panel, Efb-C–neutralizing mini-Abs cause a dose-dependent decrease of *S. aureus* growth (CFU/ml) in whole blood ("exogenous" approach). Data represent mean ± SD of three separate experiments. *p < 0.05, with reference to the 0 μmol mini-Ab value. (C) Efb secretion profile of *S. aureus* cells in whole blood. During a 12-h incubation, plasma was collected at various time points, and levels of Efb were measured by sandwich ELISA. (D) Mini-Ab treatment attenuates *S. aureus* growth (CFU/ml) in whole blood, as determined by the “endogenous” approach. The + symbol denotes the addition of each component in the reaction mixture. Data represent mean ± SD of three separate experiments. *p < 0.05. AU, arbitrary units.
diminished bacterial burden and significantly lower inflammatory scores in the kidneys, along with the prolonged therapeutic effect of a single-dose regimen in the renal abscess model all point to the further clinical exploitation of these Ab-based antimicrobial agents as leads for passive vaccination against staphylococcal infections (see also Fig. 7 for a working model of their action).

Discussion

The clinical management of *S. aureus*–associated diseases remains challenging, particularly in view of the emerging antibiotic resistance of this pathogen and the lack of targeted therapeutics that can abrogate its virulence (12, 40). In this study, we report the isolation and characterization of human mini-Abs that neutralize the function of the *S. aureus*–encoded complement inhibitory protein Efb by blocking its key interaction with complement C3 (17, 18). To the best of our knowledge, this is the first report of fully human Efb-targeting Abs that can be further exploited as vaccine leads for *S. aureus*–related diseases.

The rationale behind the selection of Efb as a target for human Ab development resonates well with its central role within the immune evasion arsenal of *S. aureus*. Efb inhibits the formation of C3b-containing convertases, thereby abrogating crucial C3-dependent effector functions that promote pathogen opsonic tagging (17). Furthermore, it appears to shield the microbe from phagocytes through the interaction of its N-terminal domain with fibrinogen and the formation of an impenetrable net over its surface (37). Thus, its broad inhibitory impact on innate immunity renders Efb an attractive target for the development of first-line therapeutic Abs that could contain bacterial growth along with antibiotics, while also affording the lymphoid compartment enough time to mount Ag-specific effector responses for the subsequent elimination of the microbe.

Applying a whole blood model of *S. aureus*–induced bacteremia, we demonstrated that Efb-C increases bacterial survival to a similar extent as the full-length protein (Efb). The absence of a similar growth-promoting effect from its nonfunctional mutant Efb-C-RENE underscored the specificity of this action and also implied that it relies on complement interception through formation of C3/Efb-C complexes.

Previous studies had attempted to measure anti-Efb Ab titers in *S. aureus* carriers (41, 42). However, due to the limited number of clinical samples, a comprehensive profile of Efb’s immunogenicity remains elusive. Our effort to systematically probe the Efb-targeted Ab profile of a large cohort of donors revealed higher Ab titers in donors classified as negative for *S. aureus*, compared with donors having active *S. aureus* infections. This somewhat counterintuitive result reflects an ongoing debate as to whether IgG responses to staphylococcal Ags such as Efb tightly correlate with acute infections or with commensal colonization and long-term nonsymptomatic carriage in the population (41, 42). Furthermore, there are disparate reports suggesting that *S. aureus*–evoked humoral responses display variability depending on frequency of exposures, strain antigenicity, and preferable site of colonization (41). Moreover, our *S. aureus*–negative cohort might include a fair proportion of nonsymptomatic carriers who have previously been
reported to present high-titer Abs. Therefore, the lack of patient stratification and long-term monitoring of anti–*S. aureus* humoral responses in our study make the interpretation of these results rather speculative. Notably, however, previous studies have suggested that high-titer Abs are stable for years in healthy individuals, thus likely conferring protective immunity against *S. aureus* (42). This latter observation prompted us to interrogate whether *S. aureus*–negative donors carry neutralizing Abs that can control *S. aureus* infections. Notably, anti-Efb Abs isolated from *S. aureus*–negative donors were capable of blocking the C3d–Efb interaction. More importantly, these Abs potentiated the neutrophil-mediated killing of *S. aureus*, suggesting that their blocking effect on the C3d–Efb interaction significantly attenuated complement activation, compromising downstream effector functions (i.e., neutrophil recruitment and phagocytosis). To circumvent several limitations and potential adverse effects (e.g., safety risks) associated with the use of IVIG in passive vaccination protocols (11), we adopted a high-throughput screening approach to generate human monoclonal Abs against Efb-C, while preserving both the diversity and magnitude of scale (e.g., 10⁹) of the human Ab repertoire (25).

The high selectivity of the resulting anti–Efb-C mini-Ab makes them promising candidates for therapeutic modulation.

**FIGURE 6.** Mini-Ab attenuation kidney abscess formation in *S. aureus*–infected mice. (A) Representative images of kidneys harvested from control (PBS-injected), *S. aureus*–infected, and infected mice pretreated with the neutralizing mini-Ab A1. Treatment with mini-Ab A1 significantly reversed the gross morphological deterioration of the organ, closely resembling the control (PBS-injected) kidneys. In contrast, the *S. aureus*–infected kidney shows prominent signs of bacterial inflammation and abscess formation, represented by yellow spots on the surface of the kidney parenchyma. (B) Mini-Ab treatment attenuates bacterial colonization in *S. aureus*–infected kidneys. Bacterial titers were measured by plating kidney homogenates. (C) Mini-Ab A1 affords protection from *S. aureus*–induced inflammation and attenuates kidney tissue damage. Top panels represent low- (×10) and bottom panels represent high-power (×40) magnification of photomicrographs from H&E-stained, paraffin-embedded kidney sections. The kidney papilla parenchyma from an *S. aureus*–infected mouse presents prominent signs of bacterial inflammation as evidenced by abscess formation and presence of bacterial communities (see bottom left panel and inset, original magnification ×100). Conversely, treatment of mice with mini-Ab A1 leads to a significant restoration of kidney histology as evidenced by the reduced extent of tissue damage and inflammation (top right panel) and the significantly reduced infiltrates containing fewer staphylococci throughout the tissue (bottom right panel). One representative image per group (*n* = 6) is shown. *p < 0.05.
of *S. aureus*’ virulence. Furthermore, the lack of an Fc portion from the mini-Ab structure confers more benefits, such as the bypass of FcγR-mediated effector pathways that may lead to faster clearance through the hepatosplenic phagocytic system, undesirable immune activation, or higher effective concentrations due to elimination of nonspecific binding to Fc receptors (10, 43). SPR-based analysis allowed the initial selection of the Abs with higher affinities toward Efb-C, and the fitting of the binding kinetics revealed a single-site mode of interaction with their ligand. However, due to their dimeric nature, each mini-Ab is expected to bind and neutralize up to two Efb molecules in solution.

Previous studies have elucidated the structural determinants of the Efb-C/C3 interaction, revealing distinct binding affinities of Efb-C for C3 and its TED-containing fragments (18, 36) and implying a binding mode that is influenced by differential accessibility to the target proteins (20). Our study revealed a panel of mini-Abs that potently inhibit the C3/Efb-C interaction. Structural studies have indicated that Efb-C residues R131 and N138, located within the binding interface of Efb-C/C3d, contribute significantly to this interaction. In search of a plausible explanation for the mini-Ab–mediated blockade of the C3d/Efb-C interaction, we hypothesized that the epitopes recognized by these Abs lie within the binding interface of the two molecules, encompassing the crucial Efb-C residues R131 and N138. Supporting this hypothesis, SPR-based analysis revealed that the blocking mini-Abs A2 and A4 most likely recognize epitopes within the binding interface, as both Abs failed to recognize the mutant Efb-C proteins. These findings strongly suggest that the inhibitory effect of the mini-Abs is largely attributed to disruption of the Efb-C/C3 complex. They also provided the basis for the subsequent evaluation of the antimicrobial activity of these mini-Abs in models of *S. aureus*–associated bacteremia.

To ascertain the neutralizing potential of these mini-Abs in a setting that closely resembles acute bacterial infection, the lead mini-Abs A1 and A2 were evaluated in a whole blood model of *S. aureus*-induced bacteremia (29). To ensure that this model represents a reliable approach for assessing the neutralizing ability of the Abs, we first tested the mini-Abs A1 and A2, adding recombinant Efb to the blood–bacteria mixture. Corroborating our hypothesis, both Abs significantly attenuated bacterial survival in a dose-dependent manner.

To date, the pattern of Efb expression in *S. aureus*-infected blood has not been determined. An early secretion profile was observed in our whole blood model, consistent with a putative role of Efb in the initial stages of bacterial infection. This early Efb secretion can be attributed to the immediate sensing of the crucial Efb-C residues R131 and N138.
phagocytic cells (Fig. 7). Interestingly, competitive binding studies revealed that mini-Ab A1 may exert a broader neutralizing effect by modulating the Efb/fibrinogen interaction, possibly via steric interference with the N-terminal domain (Supplemental Fig. 3). Notably, the pronounced therapeutic efficacy of mini-Ab A1 in our mouse model likely reflects its dual capacity to block the complement inhibitory function of Efb and also impair its ability to recruit fibrinogen to the pathogen’s surface (37). Furthermore, the lack of an Fc region raises the possibility that such mini-Abs might display a slower clearance rate, partly due to the absence of FcR-mediated interactions. Such a prolonged plasma residence coupled to a more effective diffusion within the renal tissue would likely explain their sustained therapeutic efficacy in the renal abscess model. Further studies are warranted, however, to discern the biodistribution and pharmacokinetic profile of these neutralizing mini-Abs in models of S. aureus–induced bacteremia.

In conclusion, our studies have identified novel Ab-based antimicrobial agents that exploit the complement-inhibitory protein Efb as a target for the treatment of S. aureus–induced bacteremia. Furthermore, these studies provide a rational framework for the functional refinement (i.e., affinity maturation) and comprehensive evaluation of these mini-Abs as novel immunotherapeutics in various clinically relevant models of bacterial infection.

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Disclosures
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