Antibodies against a secreted product of *Staphylococcus aureus* trigger phagocytic killing

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Host immunity against bacteria typically involves antibodies that recognize the microbial surface and promote phagocytic killing. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a frequent cause of lethal bloodstream infection; however, vaccines and antibody therapeutics targeting staphylococcal surface molecules have thus far failed to achieve clinical efficacy. *S. aureus* secretes coagulase (Coa), which activates host prothrombin and generates fibrin fibrils that protect the pathogen against phagocytosis by immune cells. Because of negative selection, the coding sequence for the prothrombin-binding D1-D2 domain is highly variable and does not elicit cross-protective immune responses. The R domain, tandem repeats of a 27-residue peptide that bind fibrinogen, is conserved at the C terminus of all Coa molecules, but its functional significance is not known. We show here that the R domain enables bloodstream infections by directing fibrinogen to the staphylococcal surface, generating a protective fibrin shield that inhibits phagocytosis. The fibrin shield can be marked with R-specific antibodies, which trigger phagocytic killing of staphylococci and protect mice against lethal bloodstream infections caused by a broad spectrum of MRSA isolates. These findings emphasize the critical role of coagulase in staphylococcal escape from opsonophagocytic killing and as a protective antigen for *S. aureus* vaccines.

Staphylococcus aureus colonizes the human nares and skin, and also causes soft tissue and bloodstream infections (David and Daum, 2010). Drug-resistant strains, designated MRSA (methicillin-resistant *S. aureus*), emerged with antibiotic use for the prevention or therapy of staphylococcal infections. MRSA infections are associated with increased failure of antibiotic therapy and increased mortality (David and Daum, 2010). To address this public health crisis, vaccines and antibody therapeutics have been developed, each targeting molecules on the staphylococcal surface, including capsule, polyglycerol phosphate lipoteichoic acid, iron-regulated surface determinant protein B (IsdB), and clumping factor A (ClfA; Spellberg and Daum, 2012). However, the corresponding clinical trials failed to reach their designated endpoints (Shinefield et al., 2002; Fowler et al., 2013).

A distinguishing feature of clinical *S. aureus* isolates is their ability to clot human plasma. This trait is based on the secretion of coagulase (Coa; Fig. 1 A; Tager, 1956), which associates with human prothrombin to form enzymatically active staphylothrombin, cleaving the A and B peptides of fibrinogen and generating fibrin fibrils (Friedrich et al., 2003). Staphylothrombin does not cut other endogenous substrates of thrombin, causing exuberant polymerization of fibrin while avoiding activation of other clotting and inflammatory factors (Panizzi et al., 2004; McAdow et al., 2012b). The resulting fibrin meshwork protects bacteria from phagocytes and is essential for the formation of *S. aureus* abscess lesions (Smith et al., 1947; Cheng et al., 2010). Activation of prothrombin is mediated by the N-terminal D1-D2 domain of Coa and blocked by specific antibodies that provide protection from *S. aureus* bloodstream infection in animal models (Rammelkamp et al., 1950; Cheng et al., 2010). Because of negative selection, *coa* is one of the most variable genes in the core genome of *S. aureus*. Up to 50% sequence variation occurs in the coding sequence for the D1-D2 domain, and the corresponding products can be categorized into serotypes without cross-protecting epitopes for the neutralization of staphylothrombin (Watanabe et al., 2009; McAdow et al., 2012a). *S. aureus* secretes a second staphylothrombin, designated von Willebrand factor binding protein (vWbp) with the conserved D1-D2 domain structure mediating association with prothrombin (Bjerketorp et al., 2004). This complex displays different catalytic activity than Coa-staphylothrombin, generating fibrin fibrils at a reduced rate and contributing to abscess formation without affecting staphylococcal escape from phagocytosis (Kroh et al., 2009; Guggenberger et al., 2012). The structural gene for vWbp, *vwb*, displays limited sequence variation, and is presumably not subject to negative selection (McAdow et al., 2012a).

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Abbreviations used: CD, cytochalasin D; Coa, coagulase; D1-D2, prothrombin binding domain of coagulase; MRSA, methicillin-resistant *Staphylococcus aureus*; OPK, opsonophagocytic killing; SK, streptokinase; vWbp, von Willebrand factor binding protein.

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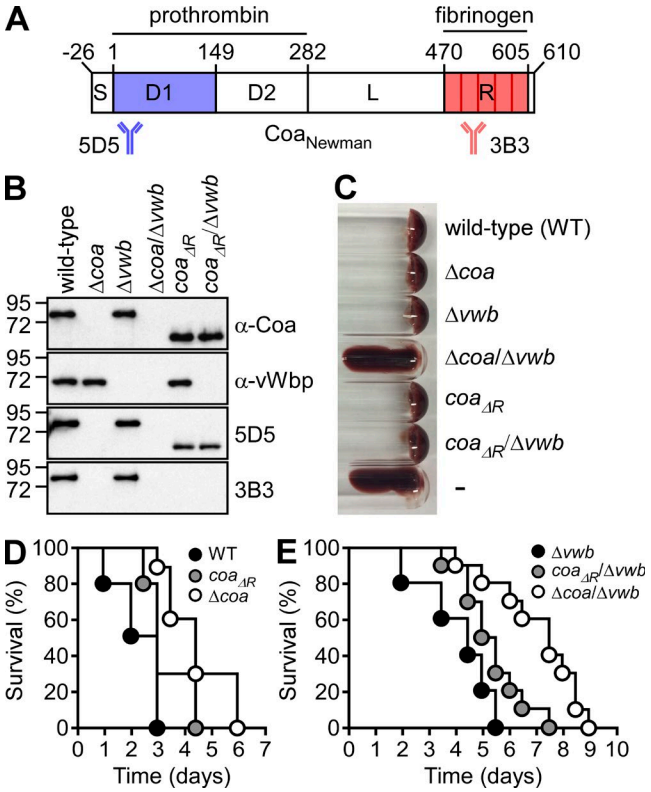

Figure 1. **The repeat domain of coagulase contributes to *S. aureus* bloodstream infections.** (A) Structure of Coa with signal sequence (S), variable D1 and D2 (prothrombin binding), linker (L), and repeat (R, fibrinogen binding) domains. The binding sites for mAbs 5D5 (blue) and 3B3 (red) are identified. (B) Secreted proteins of *S. aureus* Newman (wild-type) and coagulase variants were analyzed by immunoblotting with polyclonal α-Coa or α-vWbp and mAbs 5D5 or 3B3. Molecular weight markers (72 and 95 kD) are indicated. (C) Calcium-chelated mouse blood was inoculated with *S. aureus* strains (1×10^6 CFU) at room temperature for 24 h and coagulation was analyzed by inversion of tubes. (D and E) Mice ($n = 10$ per experiment) were challenged by intravenous injection with 8×10^7 CFU of *S. aureus* wild-type or coagulase variant strains. Data are representative of two independent analyses; (D and E) statistical significance was assessed with the log-rank test.

RESULTS AND DISCUSSION

R domain of coagulase supports *S. aureus* bloodstream infection

The C-terminal domain of Coa is conserved and comprised of tandem repeats of a 27-residue peptide which binds fibrinogen (Fig. 1 A; Watanabe et al., 2009; Panizzi et al., 2011). The number of tandem repeats varies between Coa molecules from different isolates of *S. aureus* (Watanabe et al., 2009). To characterize the contribution of the R domain to the pathogenesis of staphylococcal disease, we generated isogenic *S. aureus* variants with a truncated *coa*, lacking the R domain ($coa_{\Delta R}$), in either wild-type or Δ*vwb* backgrounds. When probed by immunoblotting with Coa- and vWbp-specific antibodies and compared with Coa from wild-type staphylococci, *S. aureus* $coa_{\Delta R}$ and $coa_{\Delta R}$/Δ*vwb* strains secreted a truncated protein into the extracellular medium (Fig. 1 B). mAb 5D5, which recognizes the D1 domain of Coa, bound to both Coa and $Coa_{\Delta R}$, whereas mAb 3B3, specific for the R domain, only bound Coa, but not $Coa_{\Delta R}$ (Fig. 1, A and B). When inoculated into calcium-chelated mouse blood and incubated for 24 h, wild-type *S. aureus* produced a firm clot, whereas mock-infected blood did not (Fig. 1 C). Staphylococci rely on secretion of both coagulases for clotting, as only Δ*coa*/Δ*vwb* but not Δ*coa* or Δ*vwb* variant strains displayed a defect in this assay (Fig. 1 C). Compared with their respective parent strains, the $coa_{\Delta R}$ and $coa_{\Delta R}$/Δ*vwb* mutants were not defective for clotting (Fig. 1 C).

When inoculated intravenously into mice, wild-type *S. aureus* Newman causes a lethal bloodstream infection within 2–3 d, whereas Δ*coa* or Δ*vwb* mutations each cause a delay in time-to-death that is additive for the Δ*coa*/Δ*vwb* mutant (median survival time 60 h [wild-type], 108 h [Δ*coa* or Δ*vwb*], and 180 h [Δ*coa*/Δ*vwb*]; Fig. 1, D and E). Surprisingly, the $coa_{\Delta R}$ mutation also caused a delay in time-to-death (median survival time 72 [$coa_{\Delta R}$] and 126 h [$coa_{\Delta R}$/Δ*vwb*]), which could be quantified in strains with (wild-type vs. $coa_{\Delta R}$, $P = 0.0308$; Δ*coa* vs. $coa_{\Delta R}$, $P = 0.0229$) or without *vwb* expression (Δ*vwb* vs. $coa_{\Delta R}$/Δ*vwb*, $P = 0.043$; Δ*coa*/Δ*vwb* vs. $coa_{\Delta R}$/Δ*vwb*, $P = 0.0084$). Thus, the R domain, although dispensable for staphylothrombin-mediated clotting, contributes to the pathogenesis of *S. aureus* infection in mice.

R domain enables assembly of the staphylococcal fibrin shield

Full-length Strep-tagged Coa (Coa_{ST}), Coa truncated for the R domain ($Coa_{\Delta R\backslash ST}$), and R domain alone ($R_{ST}$) were purified and used for affinity chromatography experiments with citrate plasma (Fig. 2 A). Coa_{ST} and R_{ST} retained molar excess of fibrinogen, whereas $Coa_{\Delta R/ST}$ retained only equimolar amounts of fibrinogen (Fig. 2 A). This can be explained by the equimolar association between fibrinogen and the exosite of staphylothrombin within Coa_{ST} or $Coa_{\Delta R/ST}$, whereas the R domain of Coa_{ST} and R_{ST} associates with 3–4 mol of fibrinogen (Fig. 2 A). As expected, Coa_{ST} and $Coa_{\Delta R/ST}$ bound prothrombin via their D1-D2 domain, whereas R_{ST} did not (Fig. 2 A). Staphylococci display surface proteins, for example ClfA, that promote association of bacteria with fibrinogen (McDevitt et al., 1994; McAdow et al., 2012a). Mixed with dilute plasma, mid-log staphylococcal cultures formed fibrin clots that, when centrifuged, sedimented with the bacteria and could be solubilized with urea (Fig. 2 B). When analyzed by Coomassie-stained SDS-PAGE, fibrin was found to be associated with the bacterial sediment, whereas albumin remained in the supernatant of agglutinated staphylococci (Fig. 2 B). Immunoblotting revealed that full-length Coa sedimented with the bacterial clot, whereas $Coa_{\Delta R}$ did not (Fig. 2 B). Association of Coa with staphylococci occurred in the presence of the fibrin clot and was not observed for staphylococcal cultures centrifuged without human plasma (Fig. 2 B). To visu-

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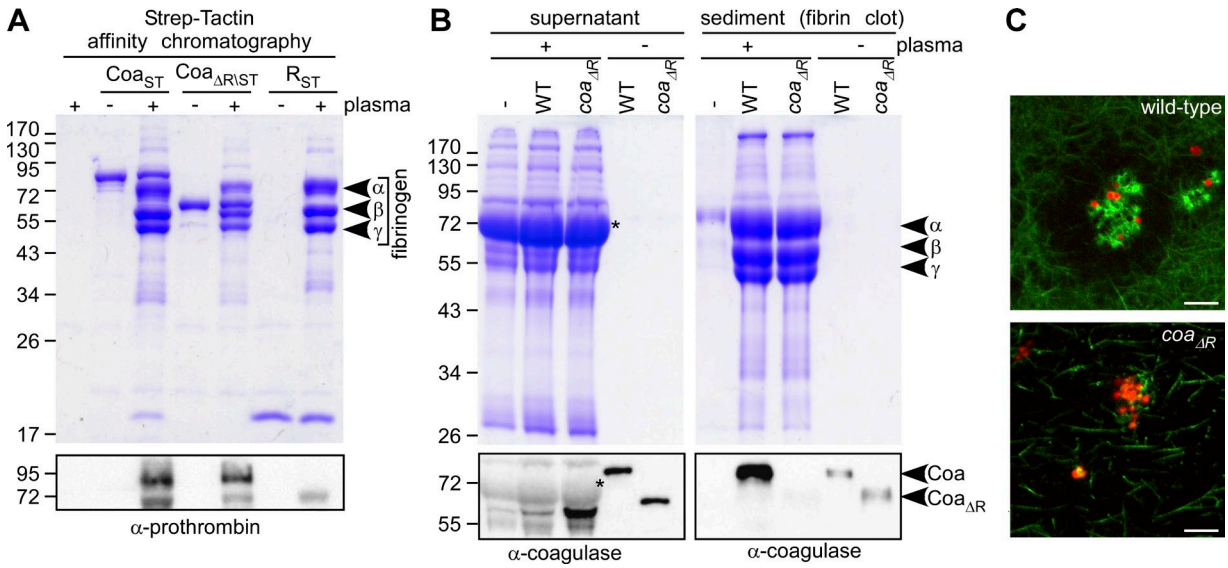

Figure 2. **The repeat domain of coagulase promotes assembly of a fibrin sheet on the surface of *S. aureus*.** (A) Human plasma (+) or PBS control (–) were subjected to chromatography on Strep-Tactin resin precharged with full-length coagulase (Coa_{ST}), coagulase truncated for the R domain ($Coa_{\Delta R/ST}$), the R domain (R_{ST}) alone, or without affinity bait. Proteins retained on the affinity column were analyzed by Coomassie-stained PAGE or immunoblotting with antibodies against prothrombin. (B) Human plasma or PBS was added to cultures of *S. aureus* wild-type, the $coa_{\Delta R}$ variant or medium control (–). Plasma proteins in the supernatant and sedimented fibrin clots were separated by centrifugation, analyzed by Coomassie-stained PAGE, or immunoblotting against Coa. Asterisks identifies albumin whose abundance affects the mobility of $Coa_{\Delta R}$. α-, β-, and γ-chains of fibrinogen/fibrin are labeled. (C) *S. aureus* wild-type or $coa_{\Delta R}$ expressing mCherry were incubated with human citrate plasma and 5% Alexa Fluor 488–conjugated human fibrinogen and imaged by fluorescence microscopy. Bars, 10 µm. Data are representative of two independent analyses.

alize the contribution of the R domain toward staphylococcal fibrin formation, mCherry-expressing bacteria were added to plasma samples with Alexa Fluor 88–conjugated fibrinogen and clot formation was viewed by fluorescence microscopy. Unlike wild-type staphylococci, which generated large fibrin deposits in the vicinity of bacteria, the $coa_{\Delta R}$ mutant produced long fibrin strands that were only loosely associated with the pathogen (Fig. 2 C). Thus, by augmenting the recruitment of soluble fibrinogen, the C-terminal repeats favor Coa-induced fibrin clots and limit diffusion of Coa away from staphylococci, thereby localizing the staphylothrombin-generated fibrin shield in the immediate vicinity of the bacteria.

R domain antibody protects mice against bloodstream infection

Mouse mAbs were raised by immunizing mice with full-length Coa of *S. aureus* Newman. 13 antibodies reactive to Coa, but not to vWbp or IsdA controls, were characterized for their affinity and specificity to D1, D2, D1-D2, D1 lacking the first 18 residues ($D1_{\Delta 1\text{-}18}$), L (linker), and R domains (Fig. 1 A). Two mAbs targeting the variable or conserved domains of Coa, 5D5 and 3B3, were used for further study. mAb 5D5, which bound to the D1 domain within the first 18 residues of D1 that insert into the prothrombin-active site to generate active staphylothrombin (Table S1), prevented Coa_{ST} binding to prothrombin but not to fibrinogen (Fig. 3, A and B). mAb 3B3, on the other hand, bound to the R domain (Table S1) and blocked Coa_{ST} association with fibrinogen but not with prothrombin (Fig. 3, A and B). Further, mAb 5D5, but not mAb 3B3, inhibited *S. aureus* Newman–mediated clotting of mouse blood in vitro; however, neither 5D5 nor 3B3 inhibited *S. aureus* agglutination of EDTA-rabbit plasma (Fig. 3, C and D). Purified mAbs 5D5 or 3B3 were injected at a concentration of 5 mg antibody/kg body weight into the peritoneal cavity of BALB/c mice and compared with IgG1 isotype control mAb (Fig. 4). Both 5D5 and 3B3 provided protection against lethal bloodstream infection with *S. aureus* Newman (IgG1 vs. 5D5, $P < 0.0001$; IgG1 vs. 3B3, $P < 0.0001$; Fig. 4 A). Similar results were obtained when the *S. aureus* Δ*vwb* variant was used as a challenge strain (IgG1 vs. 5D5, $P = 0.0011$; IgG1 vs. 3B3, $P = 0.0004$; Fig. 4 B). In ELISA assays, mAb 3B3 was observed to bind coagulase from different serotypes, including type II (Coa_{N315}), type III (Coa_{USA300}), type IV ($Coa_{MRSA252}$ and $Coa_{85/2082}$), and type VII (Coa_{WIS}; Table S2). In contrast, mAb 5D5 recognized only Coa_{USA300} and to a lesser degree Coa_{WIS} (Table S2). When analyzed for the prevention of lethal bloodstream infections, both 3B3 and 5D5 provided protection against MRSA strain USA300, with a type III coagulase similar to *S. aureus* Newman (IgG1 vs. 5D5, $P = 0.0007$; IgG1 vs. 3B3, $P < 0.0001$; Fig. 4 C). However, only mAb 3B3 protected mice against lethal bloodstream challenge with *S. aureus* N315 (IgG1 vs. 5D5, $P = 0.1186$; IgG1 vs. 3B3, $P < 0.0001$), MRSA252 (IgG1 vs. 5D5, $P = 0.5993$; IgG1 vs. 3B3, $P < 0.0001$), and MRSA isolate WIS (IgG1 vs. 5D5, $P = 0.4243$; IgG1 vs. 3B3, $P < 0.0001$; Fig. 4, D–F).

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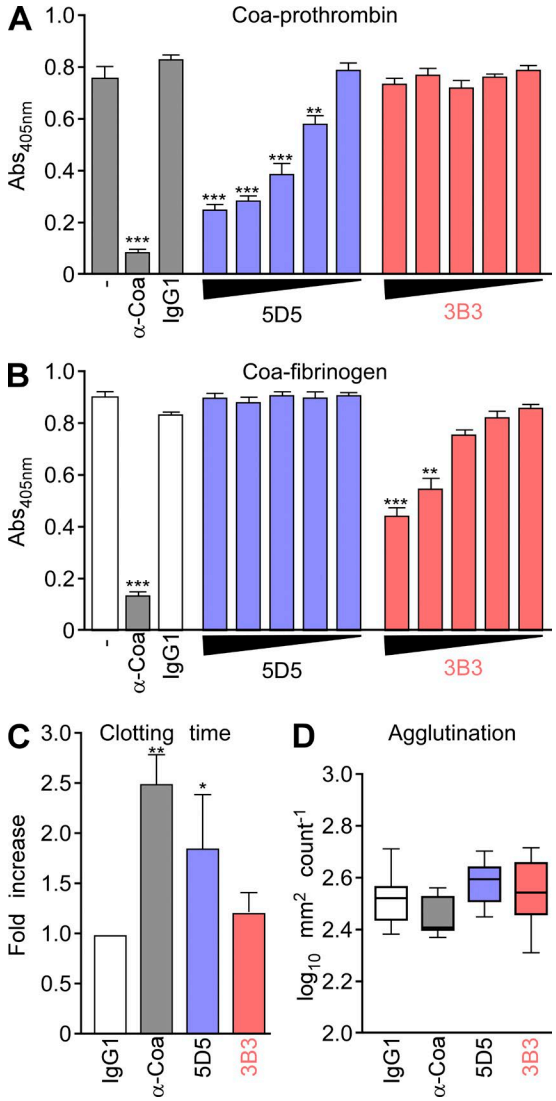

Figure 3. **Monoclonal antibodies disrupt specific activities of Coa.** (A and B) Association of Coa with human prothrombin or fibrinogen was measured by ELISA and perturbed with increasing concentrations of affinity-purified 5D5, affinity-purified 3B3, polyclonal antibodies (α-Coa), or IgG1 isotype control. (C) Calcium-chelated mouse blood was inoculated with *S. aureus* Newman (1×10^6 CFU) in the presence of 3 µM of 5D5, 3B3, polyclonal antibodies (α-Coa), or IgG1 isotype control. Samples were incubated at room temperature and monitored for coagulation. (D) Rabbit EDTA-plasma was mixed with SYTO9 stained *S. aureus* Newman (1×10^7 CFU) in the presence of 3 µM of 5D5, 3B3, polyclonal antibodies (α-Coa), or IgG1 isotype control. Samples were incubated at room temperature for 10 min, analyzed by fluorescence microscopy, and quantified by calculating means ± SEM from 12 fields of microscopic view. Statistical significance was assessed with one-way ANOVA and Bonferroni post-test. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$.

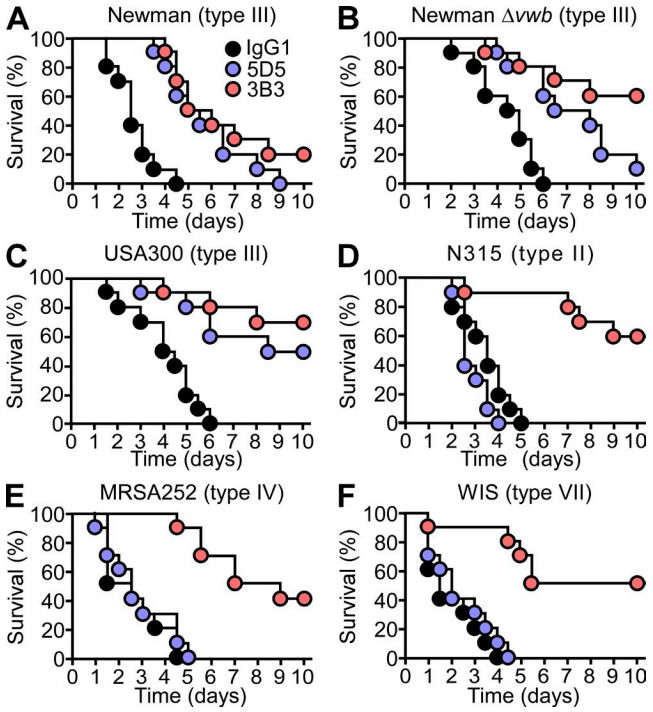

Figure 4. **Antibody against the R domain of coagulase protects against *S. aureus* bloodstream infection.** Purified mAbs 5D5, 3B3, or IgG1 isotype control were injected at a concentration of 5 mg kg^{-1} body weight into the peritoneal cavity of naive BALB/c mice. Animal cohorts (n = 10 per experiment) were challenged by intravenous injection with *S. aureus* Newman (A), the Δ*vwb* variant of Newman (B), MRSA USA300 (C), MRSA N315 (D), MRSA252 (E), or WIS (F), and survival was monitored over 10 d. Data are representative of two independent analyses; statistical significance was assessed with the log-rank test.

S. aureus agglutination in human blood

Blood from human volunteers was anticoagulated with desirudin to inhibit endogenous thrombin without affecting staphylothrombin (McAdow et al., 2011). Blood cells were removed by centrifugation, and 0.5 ml human plasma was inoculated with *S. aureus* Newman (5×10^6 CFU). Staphylococcal CFU were enumerated at timed intervals (0 and 60 min incubation at 37°C). Within 60 min, CFU for wild-type *S. aureus* dropped from 5×10^6 (100%) to 0.15×10^6 (3%), whereas CFU for the isogenic Δ*coa*/Δ*vwb* variant were not reduced (Fig. 5 A). Treatment of plasma samples with streptokinase (SK), the plasminogen activator of fibrinolysis, did not affect bacterial CFU in the 0 min samples, and liberated wild-type *S. aureus* agglutinated over 60 min (Fig. 5 A). USA300 LAC agglutinated in human plasma and replicated quickly to generate a large bacterial load. USA300 LAC agglutination did not occur in defibrinated human serum (Fig. 5 A).

S. aureus phagocytosis and opsonophagocytic killing (OPK) were measured in blood samples from 20 healthy human volunteers infected with 5×10^6 CFU USA300 LAC for 60 min. Bacterial CFU were quantified with or without SK treatment (Table S3). Control blood samples were pretreated with cytochalasin D (CD), thereby preventing *S. aureus* phagocytosis (Mimura and Asano, 1976). At a challenge dose of 10 bacteria per leukocyte, the assay quantifies OPK of

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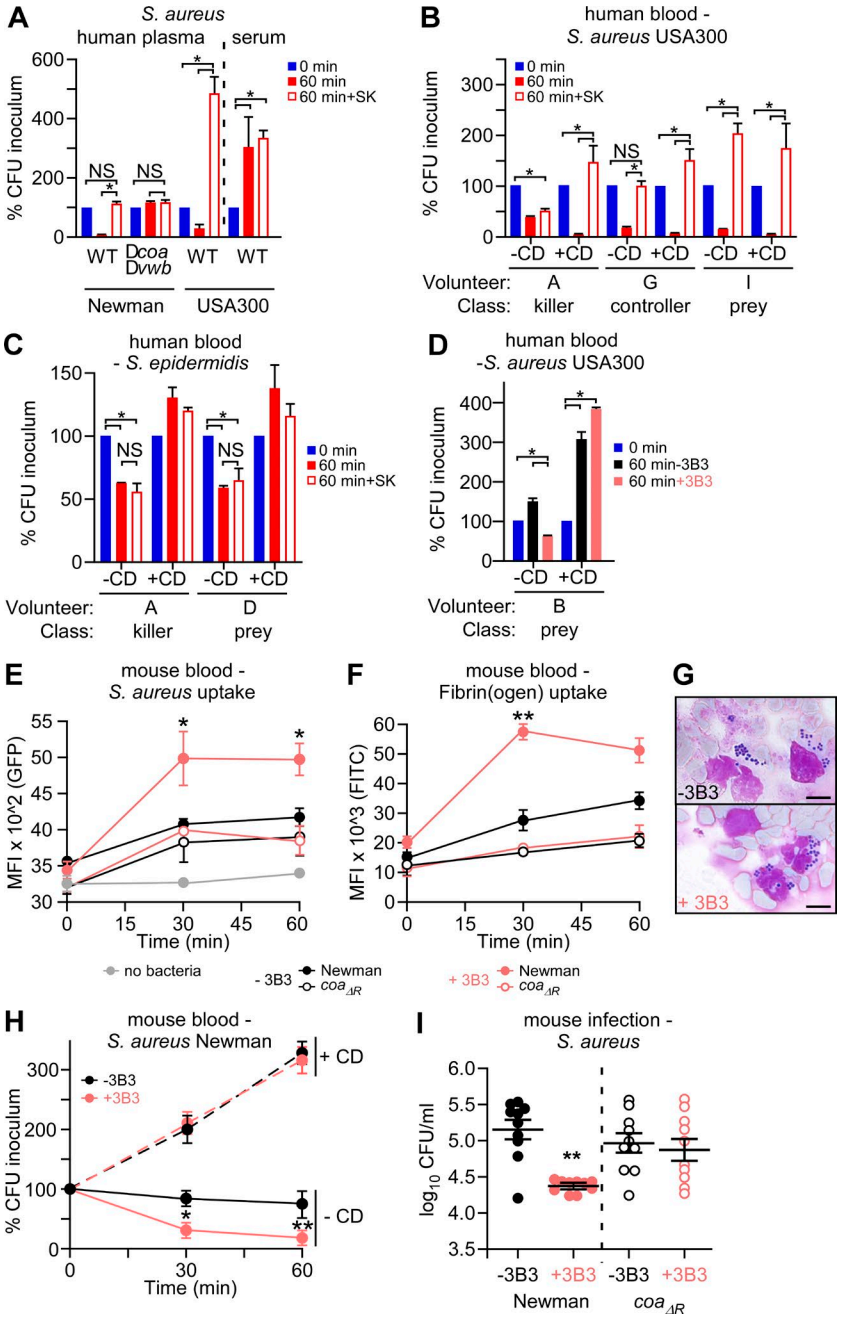

Figure 5. **Antibody against the repeat domain of coagulase promotes OPK of staphylococci.** (A) Anticoagulated human plasma or serum were inoculated with 5×10^6 CFU *S. aureus* Newman (WT), Δ*coa*/Δ*vwb*, or MRSA isolate USA300 LAC and incubated for 60 min before dilution and plating for CFU. Agglutinated staphylococci were released by SK treatment. (B and C) Anticoagulated blood from human volunteers was inoculated with 5×10^6 CFU *S. aureus* USA300 (B) or 5×10^6 CFU *S. epidermidis* (C), incubated for 60 min, and CFU enumerated with or without SK treatment. Blood samples were pretreated with CD to block phagocytosis. (D) Addition of mAb 3B3 to blood samples promoted OPK of *S. aureus* USA300. Data were collected as in B. (E) Anticoagulated mouse blood with or without mAb 3B3 was inoculated with *S. aureus* Newman (pGFP), *S. aureus* $coa_{\Delta R}$ (pGFP), or left uninfected. At 0, 30, and 60 min, extracellular bacteria were first killed with lysostaphin and neutrophils were stained with α-GR1. The mean fluorescence intensity (MFI) of GFP was used as a measure for phagocytosed bacteria. (F) Mouse blood was supplemented with 5% Alexa Fluor 488–conjugated human fibrinogen. Incorporation of Alexa Fluor 488-fibrinogen into fibrin and association with neutrophils was measured by FITC fluorescence. (G) Mouse blood was incubated for 30 min with wild-type *S. aureus* in the absence or presence of mAb 3B3, stained with Giemsa, and viewed by microscopy. Bar, 10 µm. (H) *S. aureus* Newman was incubated with anticoagulated mouse blood without or with CD and without or with mAb 3B3; staphylococcal survival and replication was assessed by CFU enumeration at timed intervals. (I) mAb 3B3 or IgG1 isotype control were administered into the peritoneal cavity of mice (n = 10 per experiment). Animals were challenged by intravenous injection with *S. aureus* Newman or the $coa_{\Delta R}$ variant. After 30 min, animals were bled via cardiac puncture and CFU enumerated. (A-D) Experiments were performed in duplicate, results averaged, and data were recorded as percent inoculum. (E, F, H, and I) Data are representative of two independent analyses conducted in triplicate. Error bars indicate SEM. Statistical analyses were performed with the two-tailed Student's t test (A–D and J) or with two-way ANOVA with Bonferroni post-test (E, F, and H). *, $P < 0.05$ and **, $P < 0.01$.

5×10^6 CFU USA300 LAC as the percent CFU reduction from 0 to 60 min in SK-treated blood. Phagocytes in blood samples of volunteer A killed 2.552×10^6 CFU (51.04%) within 60 min (Fig. 5 B). A fraction (64.62%) of the total staphylococcal load could be enumerated in blood without SK treatment (Table S3). When pretreated with CD, 97.92% of staphylococcal CFU were agglutinated in blood from volunteer A. Agglutination was calculated as the percent *S. aureus* CFU requiring SK treatment for enumeration after 60 min incubation. For volunteer A, 35.38% of the staphylococcal load had agglutinated within 60 min, whereas 64.62% had been phagocytosed (Table S3). Phagocytes in blood samples from volunteer G were unable to kill *S. aureus*: 99.68% of the inoculum was recovered in SK blood (Fig. 5 B). Here, 21.93% of the bacterial load had been phagocytosed, whereas 78.07% were agglutinated (Table S3). USA300 LAC expanded in blood samples from volunteer I to 204.42% of the initial inoculum; 85.75% of the load were agglutinated (Fig. 5 B). On the basis of these phenotypes, we categorized human blood samples as staphylococcal killer, controller, or prey (Table S3). This classification applies only to *S. aureus*, as both killer and prey blood samples were active in phagocytosis and OPK of

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Staphylococcus epidermidis, a commensal that does not express coagulases (Fig. 5 C). Antibody titers against the D1-D2 or the C-terminal R domain were not correlated with OPK of USA300 LAC in human blood (Table S3).

R domain antibody promotes phagocytosis of fibrin-coated staphylococci

When added to blood samples of volunteer B (*prey*), mAb 3B3 reduced the bacterial load to 63%, whereas USA300 LAC expanded to 128% in blood without antibody (3B3 vs. mock, $P < 0.05$; Fig. 5 D). Pretreatment of blood with CD abolished phagocytosis and OPK of USA300 LAC in the presence of mAb 3B3 (Fig. 5 D). *S. aureus* Newman–expressing GFP was inoculated into mouse blood, and neutrophils were isolated by GR1-staining and flow cytometry (Fig. 5 E). Although phagocytosis of staphylococci occurred in the absence of antibody, association of staphylococci with neutrophils was increased in the presence of mAb 3B3 (Fig. 5 E). Further, GFP fluorescence did not increase after 30 min, indicating that bacterial replication had been arrested (Thammavongsa et al., 2013). Antibody-mediated uptake of staphylococci was not observed in neutrophils from *S. aureus* $coa_{\Delta R}$ samples (Fig. 5 E). Neutrophil uptake of wild-type *S. aureus* was accompanied by uptake of fibrin, detected by adding Alexa Fluor 488–conjugated human fibrinogen to blood samples and measuring neutrophil fluorescence (Fig. 5 F). Mouse blood infected with *S. aureus* was Giemsa stained, which revealed large clumps of fibrin-agglutinated staphylococci outside of neutrophils (Fig. 5 G). When treated with mAb 3B3, staphylococci appeared to be internalized by mouse neutrophils (Fig. 5 G). Mouse blood was infected with *S. aureus* and analyzed for CFU after 30 and 60 min incubation. Compared with mock control, mAb 3B3 promoted phagocytic killing of staphylococci. As expected, OPK was blocked by pretreatment with CD (Fig. 5 H). OPK of *S. aureus* was quantified in vivo in mice with intravenous challenge of *S. aureus* followed by CFU enumeration in cardiac blood 30 min post infection. mAb 3B3 reduced the bacterial load in mice infected with wild-type *S. aureus* but not in mice infected with the $coa_{\Delta R}$ variant (Fig. 5 I).

We report that *S. aureus* evolved coagulase-mediated assembly of a fibrin shield to protect the pathogen against uptake by phagocytes. The R domain drives the formation of the bacterial fibrin shield that protects bacteria but also exposes Coa for antibody deposition. To avoid neutralizing antibody responses against Coa, coding sequence for the D1-D2 domain is subject to negative selection, generating *S. aureus* variants that are not be neutralized by antibodies against another serotype (Watanabe et al., 2009; McAdow et al., 2012a). We also show that antibody against the R domain can target staphylococci for OPK. Successful vaccines generally rely on antibodies against bacterial surface structures to implement pathogen destruction (Robbins et al., 1996). *S. aureus* escapes antibody-mediated destruction by a number of different immune evasion mechanisms, blocking neutrophil chemotaxis, phagocytosis, complement activation, and antibody deposition (Spaan et al., 2013). To address this, vaccine developers rely on a standardized assay, measuring OPK in cultured HL60 phagocytes supplemented with complement and antibody but not with hemostasis factors (Nanra et al., 2013). This assay does not, however, assess the immune evasive attributes of coagulase and may overestimate the role of antibodies in promoting OPK.

MATERIALS AND METHODS

Bacterial growth, strains, and plasmids. *S. aureus* and *Escherichia coli* were grown in tryptic soy and Luria broth or agar, with ampicillin (100 µg ml^{-1}) or chloramphenicol (10 µg ml^{-1}) when necessary. Earlier work reported *S. aureus* Newman and its variants Δ*coa*, Δ*vwb*, and Δ*coa*/Δ*vwb* with or without plasmid expressing GFP or mCherry (Cheng et al., 2010). pKOR1 was used to introduce the $coa_{\Delta R}$ allele (deletions of codons 470–605) into wild-type or Δ*vwb* Newman (Bae and Schneewind, 2006). Earlier work generated *E. coli* plasmids for purification of full-length mature Coa (*S. aureus* Newman, USA300, N315, MRSA252, 85/2082, or WIS; McAdow et al., 2012a; Thomer et al., 2013) or Coa Newman domains (D1, D1-D2, $D1_{\Delta 1\text{-}18}$, D2, and L; McAdow et al., 2012a). Plasmid pET15b-r_{ST} harbors coding sequence for the R domain (codons 470–605) and a C-terminal Strep tag.

Identification of coagulases in cultures and clots. To examine the secretion of coagulases, cultures of staphylococci were grown to an optical density A_{600} 0.4 (~10^8 CFU ml^{-1}). Proteins in the supernatant, i.e., 1 ml of centrifuged culture, were precipitated with 75 µl of trichloroacetic acid 100% (wt/vol), washed with acetone, dried, and solubilized in 50 µl sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue). To examine the fate of coagulase in fibrin clots, 950 µl of bacterial culture (~10^8 CFU ml^{-1}) or broth were mixed with 50 µl of PBS or human citrate plasma for 10 min at 37°C and centrifuged at 13,000 *g* for 10 min to separate soluble and clotted materials. 4 M urea was used to solubilize fibrin clots before separation of extracts by SDS-PAGE. Proteins were visualized with Coomassie staining or transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting using rabbit affinity-purified antibodies against Coa (α-Coa) or vWbp (α-vWbp; Thomer et al., 2013) and mouse affinity-purified monoclonal antibodies 3B3 or 5D5.

Pull down experiments. Coa_{ST}, $Coa_{\Delta R/ST}$, and R_{ST} were purified over Strep-Tactin-Sepharose (IBA) following methods described earlier for Coa subdomains and Coa strain variants (McAdow et al., 2012a; Thomer et al., 2013). All purified proteins were stored in PBS. For pull-down experiments, citrate plasma from healthy human volunteers (500 µl) diluted 1:1 in PBS was applied by gravity flow over Strep-Tactin-Sepharose beads precharged or not with 100 nmol of purified Coa_{ST}, $Coa_{\Delta RST}$, or R_{ST}. Bound proteins were recovered by boiling

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the resin in sample buffer and analyzed by SDS-PAGE separation followed by Coomassie staining or immunoblot.

Coagulation assay. 10 µl of bacterial suspension (~10^8 CFU ml^{-1}) was added to 90 µl of freshly collected mouse blood anticoagulated with sodium citrate (10 mM final concentration) in a sterile plastic test tube (BD). Samples were incubated at room temperature, and blood coagulation was verified by tipping the tubes to 45° angles at timed intervals. Where indicated, antibodies were added at a final concentration of 3 µM. Statistical analysis was performed by two-tailed Student's *t* test using Prism (GraphPad Software).

Microscopy. For visualization of bacteria in clots, 5 µl of staphylococci expressing mCherry (~10^8 CFU ml^{-1}) were mixed for 5 min with 5 µl of human citrate plasma supplemented with 5% Alexa Fluor 488–conjugated human fibrinogen (Life Technologies). Images of samples placed on glass slides were captured on a SP5 tandem scanner spectral 2-photon confocal microscope (Leica) using a 100× objective. For assessment of agglutination, 1 ml staphylococci (~10^8 CFU ml^{-1}) was incubated with 1:500 SYTO9 (Invitrogen) for 15 min, washed twice, and suspended in 1 ml PBS. Bacteria were incubated 1:1 for 15 min with human citrate plasma on glass microscope slides. Where indicated, antibodies were added at a final concentration of 3 µM. Images were captured on a live cell total internal reflection fluorescence microscope (IX81; Olympus) using a 20× objective. The threshold function in ImageJ software (National Institutes of Health) was used to convert the image into a dichromatic format in which staphylococci are black and the background is white. Statistical significance was determined by two-way analysis of variance using Prism.

Production of monoclonal antibodies against coagulase. Three 8-wk-old BALB/c female mice (The Jackson Laboratory) were immunized by intraperitoneal injection with 100 µg of purified recombinant Coa_{NM} emulsified 1:1 in Complete Freund's Adjuvant (DIFCO) for the first immunization. On days 21 and 42, animals were boosted with 100 µg Coa_{NM} emulsified 1:1 in Incomplete Freund's Adjuvant (DIFCO). On days 31 and 52, animals were bled and screened by ELISA on MaxiSorp (Nunc) 96-well flat bottom plates coated with Coa. 79 d after the initial immunization, mice that showed strong immunoreactivity to antigen were boosted with 25 µg Coa in PBS. 3 d later, splenocytes were harvested and fused with the mouse myeloma cell line SP2/mIL-6, an IL-6–secreting derivative of SP2/0 myeloma cell line. Hybridomas were screened by ELISA and antigen-specific clones subcloned by limiting dilution to produce monoclonal antibody-secreting hybridomas arising from single cells. Hybridoma cell lines were grown until a density of 10^6 cells ml^{-1} in DMEM-10 medium with 10% FBS and left spending for 6 wk. Antibodies were purified from filtered culture supernatants by affinity chromatography as previously described (McAdow et al., 2012a; Thomer et al., 2013).

ELISA. To determine the binding affinity and specificity of mAbs, Nunc MaxiSorp 96-well plates were coated with the various Coa variant serotypes and subdomains prepared at a concentration of 20 nM in 0.1 M sodium bicarbonate and affinities were measured as described earlier (McAdow et al., 2012a). ELISA plates coated with vWbp and IsdA served as negative controls. The ability of mAbs to interfere with the binding of prothrombin or fibrinogen was measured as described previously (McAdow et al., 2012a), and statistical analyses were performed using one-way ANOVA with Bonferroni post-test. Half-maximal IgG titers in serum from human volunteers for binding to purified Hla, $D1\text{-}D2_{ST}$, or R_{N12D} were determined by ELISA as described previously (McAdow et al., 2012a). R_{N12D} is a translational hybrid between SpA_{KKAA}, a variant of SpA that does not bind immunoglobulin, and two 27-residue repeats of the R domain from Coa_{Newman}, with $Asn^{12}Asp$ at position 12 of each repeat, followed by a C-terminal Strep tag; purified R_{N12D} for is defective fibrinogen binding.

Animal infection and immunization studies. 6-wk-old female BALB/c mice (cohorts of 10; Charles River) anesthetized with 100 mg ml^{-1} ketamine and 20 mg ml^{-1} xylazine per kilogram of body weight were inoculated into the periorbital venous plexus with 100 µl of bacterial suspension in PBS at a concentration of 2×10^8 CFU ml^{-1} (USA300), 8×10^8 CFU ml^{-1} (Newman, N315, WIS), or 2×10^9 CFU ml^{-1} (MRSA252). mAbs were injected at a concentration of 5 mg kg^{-1} into the peritoneal cavity 10 h before challenges. Statistical analyses were performed by two-tailed log-rank test using Prism. To assess the fate of staphylococci in blood (in vivo blood survival assay), animals were euthanized by CO_2 inhalation 30 min after infection and cardiac puncture was performed. Blood samples were treated with 0.5% saponin to lyse eukaryotic cells, serially diluted in PBS, and plated on agar for enumeration of CFU. Statistical analysis was performed using two-tailed Student's *t* test. Animal experiments were performed in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Chicago.

Bacterial survival in blood, opsonophagocytosis assay, and flow cytometry analysis. To measure bacterial replication and survival ex vivo, 0.5 ml of freshly drawn mouse or human blood anticoagulated with 0.005 mg desirudin per ml was incubated with 50 µl of a bacterial suspension containing 5×10^5 CFU (mouse) or 5×10^6 CFU (human). Where indicated, human blood was processed to generate desirudin-plasma or serum. Where indicated, 5% Alexa Fluor 488–conjugated human fibrinogen (Life Technologies), CD (0.04 mM), or purified mouse monoclonal antibodies (~10 µg ml^{-1} final concentration) were added to the samples. After incubation at 37°C for 0, 30, or 60 min, 0.5 ml of PBS with 0.5% saponin or 0.5 ml agglutination lysis buffer (0.5% saponin, 200 U

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SK K, 100 µg trypsin, 2 µg DNase, 10 µg RNase per ml PBS) were added to each sample for 10 min at 37°C before plating on agar for enumeration of CFU. Treatment with agglutination lysis buffer is annotated as +SK in the figures. Statistical analysis was performed by two-tailed Student's *t* test. For flow cytometry analysis, samples were incubated first with lysostaphin (10 µg ml^{-1}) for 5 min to lyse extracellular bacteria and next with erythrocyte lysis buffer (QIAGEN) for 30 min on ice. Blood leukocytes were recovered after centrifugation at 400 *g*, washed three times, and suspended in PBS containing 1% FBS. Cells were stained with allophycocyanin-conjugated α-GR1 and analyzed using a FACSCanto (BD). The data were analyzed with the two-tailed Student's *t* test. Human volunteers were enrolled under a protocol that was reviewed and approved by the University of Chicago's Institutional Review Board.

Online supplemental material. Table S1 shows the binding sites and affinities of mAbs 5D5 and 3B3 for coagulase from *S. aureus* Newman. Table S2 shows the affinity of mAbs 5D5 and 3B3 for coagulases from different clinical isolates of *S. aureus*. Table S3 analyzes agglutination, phagocytosis, and OPK of *S. aureus* in blood samples from 20 human volunteers. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20150074/DC1.

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