BACTEREMIA

Repurposed drugs block toxin-driven platelet clearance by the hepatic Ashwell-Morell receptor to clear *Staphylococcus aureus* bacteremia

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Staphylococcus aureus **(SA) bloodstream infections cause high morbidity and mortality (20 to 30%) despite modern supportive care. In a human bacteremia cohort, we found that development of thrombocytopenia was correlated to increased mortality and increased α-toxin expression by the pathogen. Platelet-derived antibacterial peptides are important in bloodstream defense against SA, but α-toxin decreased platelet viability, induced platelet sialidase to cause desialylation of platelet glycoproteins, and accelerated platelet clearance by the hepatic Ashwell-Morell receptor (AMR). Ticagrelor (Brilinta), a commonly prescribed P2Y12 receptor inhibitor used after myocardial infarction, blocked α-toxin–mediated platelet injury and resulting thrombocytopenia, thereby providing protection from lethal SA infection in a murine intravenous challenge model. Genetic deletion or pharmacological inhibition of AMR stabilized platelet counts and enhanced resistance to SA infection, and the anti-influenza sialidase inhibitor oseltamivir (Tamiflu) provided similar therapeutic benefit. Thus, a "toxin-platelet-AMR" regulatory pathway plays a critical role in the pathogenesis of SA bloodstream infection, and its elucidation provides proof of concept for repurposing two commonly prescribed drugs as adjunctive therapies to improve patient outcomes.**

INTRODUCTION

Staphylococcus aureus (SA) is one of the most important human bacterial pathogens, as the second leading cause of bloodstream infections (bacteremia) and the leading cause of infective endocarditis (*1*). Despite modern supportive measures, overall mortality in SA bacteremia has not declined in decades and remains unacceptably high (20 to 30%), with substantial risk of complications including sepsis syndrome, endocarditis, and metastatic foci of infection (for example, osteomyelitis) (*2*). High-risk populations include the elderly, diabetics, and surgical and hemodialysis patients (*3*). Multidrug resistance [such as with methicillin-resistant SA (MRSA)] is prevalent and associated with adverse outcome and increased medical costs (*4*).

The high incidence of SA bacteremia signifies a remarkable capacity of the organism to resist host innate defense mechanisms that function to prevent pathogen bloodstream dissemination (*5*). Extensive research has focused on SA virulence factors that counteract opsonization by serum complement (*6*), surface-anchored protein A that impairs the Fc function of antibodies (*7*), and the pathogen's numerous resistance mechanisms to avoid phagocytosis and oxidative burst killing by neutrophils (*8*).

Comparatively less is understood about how SA interacts with circulating platelets. These abundant, small anucleate cells are best known for their central role in hemostasis but are increasingly appreciated to have bioactivities relevant to immune defense (*9*). Platelets can act as mechano-scavengers to bundle bacteria (*10*) and enhance the function of professional phagocytic cell types such as neutrophils (*11*), macrophages (*12*), and hepatic Kupffer cells (*13*, *14*). Platelets express several Toll-like receptors that recognize pathogen-associated molecular patterns (*15*) to activate their release of proinflammatory cytokines [for example, interleukin-1β (IL-1β)] (*16*) and antimicrobial peptides including platelet microbicidal protein (tPMP) and human β-defensin-1 (hBD-1) with direct antibacterial actions (*12*, *17*, *18*). SA activates platelets via integrin GPIIb/IIIa, FcγRIIa receptor, and A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10)–dependent pathways (*19*–*21*), and the pathogen induces platelet aggregation via its "clumping factors" ClfA and ClfB (*22*, *23*).

In a cohort of 49 patients with SA bacteremia, we report a strong association of mortality with lowered platelet count (thrombocytopenia) rather than changes in leukocyte count. Platelet depletion in mice was recently shown to impair SA clearance (*12*, *24*). Our mechanistic analysis with human platelets ex vivo and murine platelets in vivo revealed a critical activity of platelets in direct killing of SA. The pathogen attempts to counteract this defense by deploying a pore-forming toxin (α-toxin) to both disrupt platelet antimicrobial activity and accelerate sialidase-dependent platelet clearance through the hepatic Ashwell-Morell receptor (AMR). Elucidation of this "toxin-platelet-AMR" regulatory pathway guided us to therapeutic repurposing of two U.S. Food and Drug Administration (FDA)–approved drugs to preserve platelet homeostasis, thereby providing significant host protection in experimental SA bloodstream infection.

RESULTS

Platelets are essential for blood immunity against SA bacteremia

The normal human platelet count ranges from 150,000 to 450,000/mm^3 of blood. In 49 consecutive patients with SA bacteremia [blood culture

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growing MRSA or MSSA (methicillin-sensitive SA)] identified prospectively at an academic medical center in Madison, WI (*25*), we observed a strong association of patient mortality with thrombocytopenia (platelet count <100,000/mm^3) on the initial blood sample and without abnormally elevated or reduced leukocyte count (Fig. 1, A and B). Two patients with thrombocytopenia failed to clear their bacteremia despite antibiotic therapy for more than 60 days before succumbing. No significant correlation was observed between platelet count and serum concentrations of proinflammatory cytokine IL-1β or APACHE score, a clinical metric of disease severity (Fig. 1A). These data are consistent with a previous single center study in Israel, showing thrombocytopenia (and not leukocyte count) to be a significant risk factor for 30-day all-cause mortality in SA bacteremia [unadjusted odds ratio (OR), 2.41; 95% confidence interval (CI), 1.76 to 3.32; $P < 0.001$], although overall mortality rates were much higher than other published series (56.2% in thrombocytopenic group versus 34% with normal platelet counts) (*26*). These clinical studies suggest that circulating platelets, and not white blood cells, could play the dominant role in resolution of SA during bloodstream infection. When directly compared with the second most common Gram-positive bacterial pathogen associated with human bacteremia, *Streptococcus pneumoniae* (*1*), SA was 49.3% more resistant to killing by purified human neutrophils, but 63.7% more susceptible to killing by human platelets (Fig. 1C).

We pursued this association further using an in vivo model of SA bacteremia (MRSA strain USA300 TCH1516) established by intravenous tail vein injection in mice, where normal platelet count ranges from 900,000 to 1.4 million cells/mm^3 blood (*27*). Within 2 hours of SA challenge, the circulating platelet count of infected mice was reduced by 37.6% from baseline (1237 ± 49.82 versus 772 ± 49.91) (Fig. 1D). To verify that reduced platelet count was indicative of a functional immune deficiency, we used an anti-CD41 antibody to deplete mice of platelets to 17% of baseline abundance (fig. S1). The drawn blood of the thrombocytopenic animals was impaired in ex vivo killing of SA [67.5 ± 7.9% surviving CFU (colony-forming unit) versus 25 ± 2.1% surviving CFU in normal blood] (Fig. 1E), and bacterial burdens in blood and kidneys of thrombocytopenic mice were significantly ($P < 0.05$) increased versus untreated mice within 2 hours after intravenous SA challenge (Fig. 1F).

SA α-toxin induces thrombocytopenia to evade platelet-mediated microbicidal activity

A major SA secreted virulence factor, the pore-forming α-toxin (Hla), induces platelet cytotoxicity and aberrant aggregation after binding its protein receptor ADAM-10 on the platelet surface (*14*, *20*, *28*). Using ImageJ densitometric analysis of anti-Hla Western immunoblots, we grouped the SA bacteremia isolates from our clinical cohort into low ($n = 18$), medium ($n = 15$), and high ($n = 16$) α-toxin producers (fig. S2). A significant association ($P < 0.05$) was seen between thrombocytopenia and high-level α-toxin production (Fig. 1G) but not the low- or medium-level α-toxin production. There was no significant association between α-toxin production and leukocyte counts (Fig. 1H). For comparison to the wild-type (WT) parent SA strain in analyses of platelet interactions, we constructed a ΔHla knockout strain by precise allelic replacement (*29*) of its encoding gene *hla* (fig. S3). In the mouse intravenous challenge model, the SA ΔHla mutant induced less thrombocytopenia (Fig. 1I) and was more rapidly cleared from the blood circulation (Fig. 1J) than the WT parent strain. Ex vivo, the SA ΔHla mutant was more susceptible to killing by purified human platelets (Fig. 1K). Together, these studies indicate that the virulence effects of SA α-toxin extend to evasion of direct platelet-mediated antibacterial killing.

FDA-approved P2Y12 inhibitor ticagrelor blocks SA α-toxin–mediated platelet cytotoxicity

Inhibition of platelet activation is the target of antithrombotic drug therapy designed to reduce the risk of cardiovascular death, myocardial infarction (MI), and stroke in patients with acute coronary syndrome or a history of MI, beginning with classical studies of aspirin [acetylsalicylic acid (ASA)] in the 1970s, then extending to newer selective inhibitors of adenosine signaling through the platelet P2Y12 receptor (clopidogrel, prasugrel, and ticagrelor) (*30*). However, the effect of "antiplatelet" drugs on the direct antibacterial properties of platelets has not been reported. Of potential relevance, a clinical study of 224 consecutive patients with community-acquired pneumonia found that those receiving antiplatelet therapy (ASA and/or clopidogrel) for secondary prevention of cardiovascular disease reduced need for intensive care unit (ICU) treatment (OR, 0.19; 95% CI, 0.04 to 0.87) and shorter hospital stays (13.9 ± 6.2 versus 18.2 ± 10.2 days) compared to their age-matched cohort (*31*). Additional human retrospective or matched cohort studies of endocarditis, bacteremia, or sepsis (not restricted to SA) have provided similar hints of improved clinical outcome among patients receiving antiplatelet drugs (*32*–*35*).

To discriminate the effect of the two antiplatelet drug classes on SA killing, we pretreated freshly isolated human platelets for 15 min with ASA or ticagrelor (chosen because clopidogrel is a prodrug requiring hepatic conversion in vivo) and coincubated them with the bacteria. Within 2 hours, ticagrelor-treated platelets showed a 2.2-fold enhancement of SA killing versus untreated controls (Fig. 2A), whereas ASA did not significantly alter platelet antibacterial activity. In contrast, ticagrelor did not promote macrophage or neutrophil killing of SA, did not alter neutrophil extracellular trap production, and did not directly inhibit SA growth (fig. S4, A to D). Upon direct coincubation of SA with human platelets in a tissue culture well, severe platelet damage was evident by transmission electron microscopy (TEM); however, ticagrelor treatment preserved platelet structural integrity against SA-induced injury (Fig. 2B). As α-toxin is the principal driver of SA platelet toxicity, we measured α-toxin–induced lactate dehydrogenase (LDH) release from platelets treated with ticagrelor, ASA [cyclooxygenase-1 (COX-1) inhibitor], or specific small-molecule inhibitors of other known platelet activation receptors [CD41, protease-activated receptor-1 (PAR-1), and PAR-4]. Among these agents, only ticagrelor significantly inhibited SA α-toxin–induced platelet LDH release (Fig. 2C), doing so in a dose-dependent manner (Fig. 2D). The deleterious effect of α-toxin on platelets involves activation of its receptor protease ADAM-10, leading to intracellular Ca^{2+} mobilization (*20*), and these biological effects were both inhibited by ticagrelor as measured in specific assays (Fig. 2, E and F). Whereas ticagrelor did not alter the amount of ADAM-10 expressed on the platelet surface (Fig. 2G), the P2Y12 inhibitor drug blocked SA-induced ADAM-10–dependent shedding of platelet Glycoprotein VI (GP6) (Fig. 2H). SA exposure also induced P-selectin, a transmembrane protein specific to α granules that is translocated to the platelet surface upon activation (Fig. 2I), and increased surface expression of CD63, a marker of dense granule mobilization (fig. S5A). The SA-induced up-regulation of P-selectin and CD63 was blocked upon ticagrelor treatment (Fig. 2I and fig. S5A).

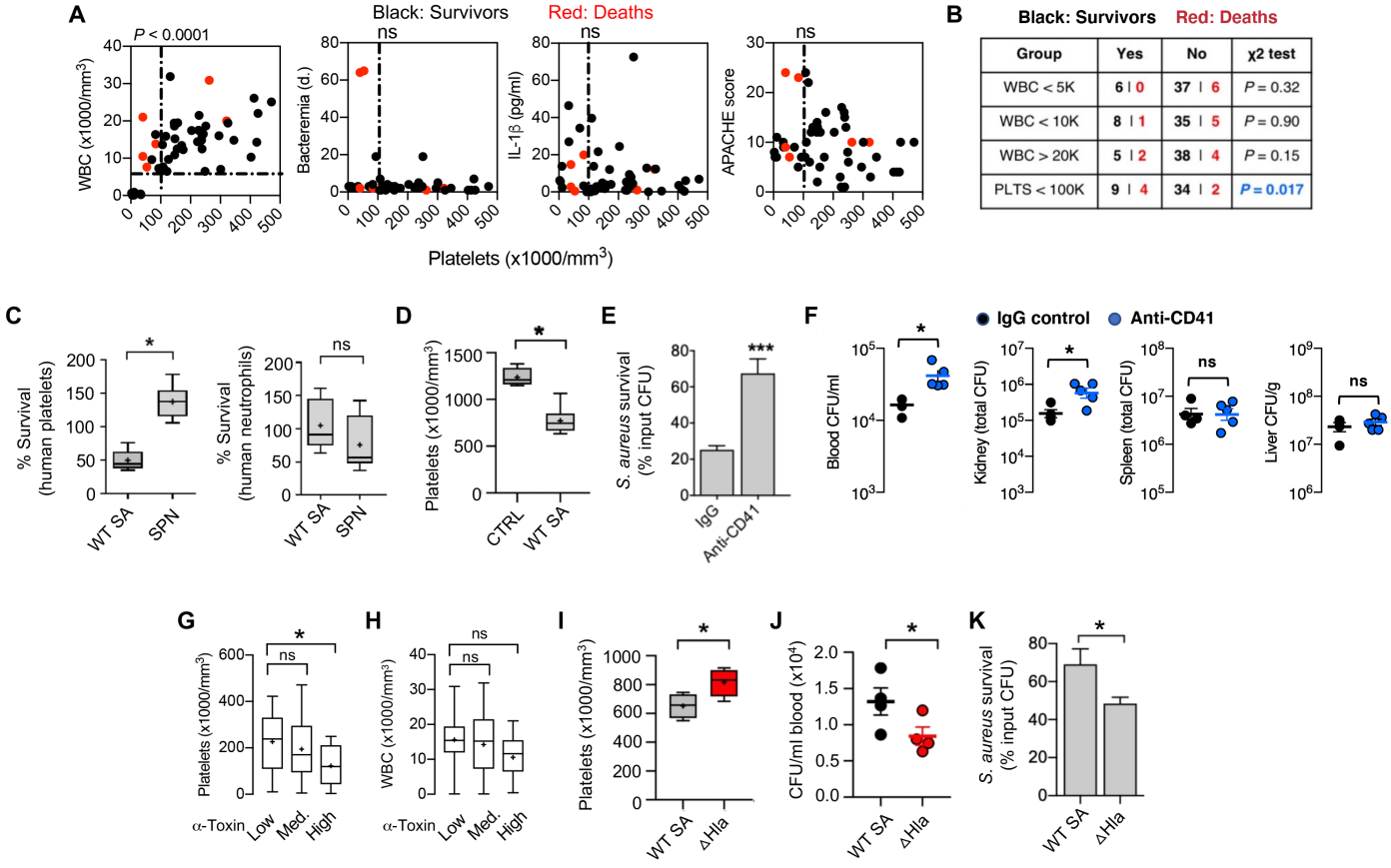

Fig. 1. Platelets are essential for blood immunity against SA bacteremia, and SA α-toxin induces thrombocytopenia to evade platelet-mediated microbicidal activity. (**A**) Correlation of circulating platelet counts with leukocyte counts and duration of bacteremia in 49 consecutive patients with SA bacteremia from a tertiary medical center. Spearman's rank correlation coefficient compared variables. (**B**) Mortality in patient cohort associated with different white blood cell (WBC) and platelet (PLTS) count cutoffs; chi-square without Yates correction. (**C**) Washed isolated human platelets and neutrophils exposed to SA or *S. pneumoniae* (SPN) at a multiplicity of infection (MOI) of 0.01 for 2 hours. Samples were sonicated, serially diluted, and plated on THA plates for enumeration of bacterial CFU. (**D**) Reduction in platelet count 2 hours after intravenous infection of mice with SA ($n = 8$) versus noninfected littermate controls ($n = 4$). Two biological replicates were performed, and data were pooled; data are represented as mean ± SEM. (**E**) Ex vivo killing of SA upon 2-hour coincubation with blood collected from mice 16 hours after treatment with anti-CD41 antibody ($n = 9$) or immunoglobulin (IgG) control ($n = 12$). (**F**) Mice treated with platelet-depleting anti-CD41 antibody ($n = 5$) or IgG control ($n = 4$) for 16 hours before intravenous SA infection. Organs were harvested and CFU was enumerated 2 hours after infection in triplicate for each sample. (**G**) Assessment of α-toxin production by the infecting SA isolate in 49 consecutive bacteremia cases and its association with patient platelet counts and (**H**) white blood cell counts. (**I** and **J**) Platelet counts (I) and CFUs (J) in outbred CD-1 mice intravenously challenged with WT SA ($n = 4$) or isogenic ΔHla mutant ($n = 4$). Blood was harvested by cardiac puncture, complete blood count was performed, and CFUs were enumerated 4 hours after infection. (**K**) Ex vivo killing of SA by freshly isolated human platelets (2-hour coincubation) versus isogenic ΔHla mutant. All data are represented as mean ± SEM and are representative of at least three independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t* test (C to F and I to K) or one-way ANOVA with Bonferroni's multiple comparisons test (G and H). *$P < 0.05$. For floating bar graphs, + denotes the mean, whiskers represent minimum to maximum, and floating box represents 25th to 75th percentile. ns, not significant.

Neither SA nor ticagrelor produced a statistically significant change in platelet β-galactosidase activity, another lysosomal marker ($P > 0.05$ for both; fig. S5B).

FDA-approved P2Y12 inhibitor ticagrelor protects against SA bacteremia

Inhibition of α-toxin–mediated platelet cytotoxicity suggested that P2Y12 inhibition using ticagrelor could mitigate the toxin's virulence role in driving SA-induced thrombocytopenia to promote bloodstream survival of the pathogen. Intravenous SA challenge in mice drove down platelet counts beginning as early as 4 hours (35% decrease, $P < 0.0005$) and continuing through 24 hours (63% decrease, $P < 0.0005$), with partial recovery by 72 hours (35% decrease, $P < 0.005$) (fig. S6A); bone marrow analysis at 72 hours revealed increased thrombopoiesis as evidenced by greater megakaryocyte number and by ploidy distribution (fig. S6, B and C). The rapid SA-induced thrombocytopenia was associated with platelet GP6 shedding (fig. S6, D and E) and platelet microparticulation (fig. S6F), the latter determined by in vitro studies to be α-toxin dependent (fig. S6G). Mice treated with ticagrelor maintained higher numbers of circulating platelets compared to control animals after SA intravenous infection (Fig. 3A), significantly reducing the bacterial burden in the blood ($P < 0.05$, Fig. 3B) and in systemic organs (kidney $P < 0.005$, liver $P < 0.0005$, spleen $P < 0.0005$; Fig. 3C), ultimately improving survival in a 10-day mortality study (Fig. 3D). Blinded histological examination of tissues by a veterinary pathologist revealed treatment-associated differences in the kidneys (Fig. 3E) and the heart (fig. S7). A 4- to 10-fold reduction in bacterial

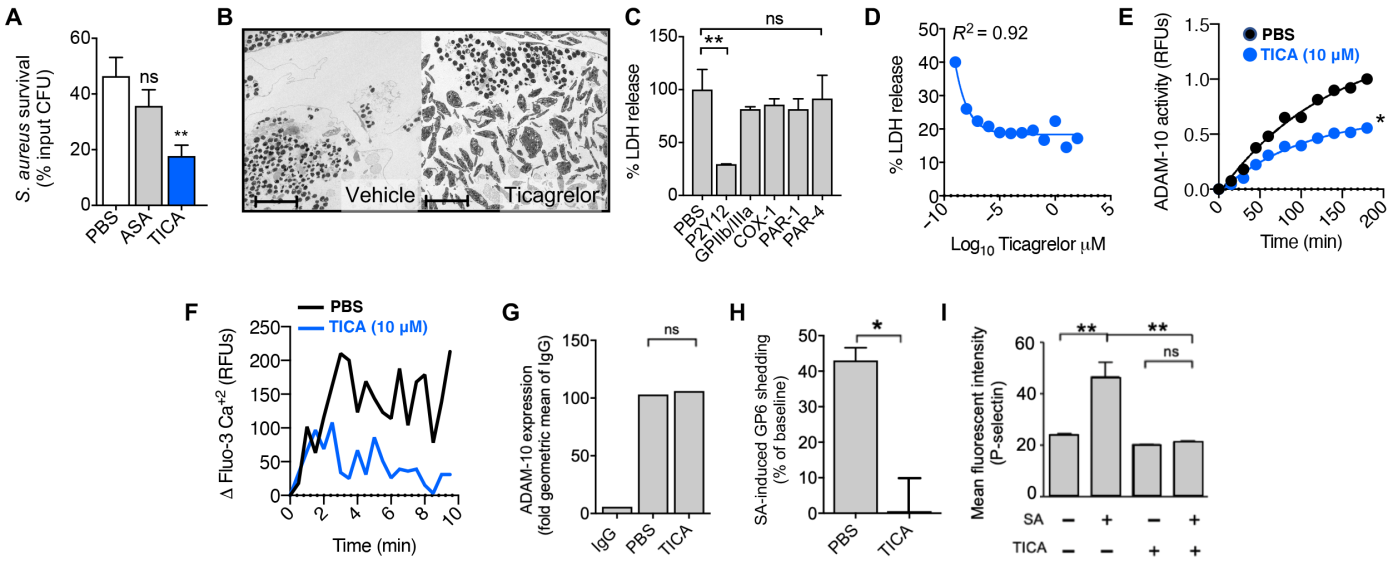

Fig. 2. FDA-approved P2Y12 inhibitor ticagrelor blocks SA α-toxin–mediated platelet cytotoxicity. (**A**) Effect of 10 μM aspirin (ASA) or 10 μM ticagrelor (TICA) (15-min pretreatment ex vivo) on human platelet killing of MRSA for 2 hours ($n = 9$). Experiments were performed in triplicate and repeated three times. (**B**) Representative transmission electron microscopy image of platelets pretreated with or without 10 μM ticagrelor and exposed to MRSA at MOI 0.1 for 2 hours. Scale bar, 5 μm. (**C**) P2Y12 inhibitor (ticagrelor) pretreatment blocks human platelet cytotoxicity by purified α-toxin (5 μg/ml) as measured by LDH release ($n = 3$) in a (**D**) dose-dependent manner. Inhibitors: P2Y12 (ticagrelor), GPIIb/IIIa (eptifibatide), COX-1 (SC560), PAR-1 (vorapaxar), and PAR-4 (ML-354). (**E**) Ticagrelor (blue line) treatment of human platelets reduces proteolytic cleavage of an ADAM-10–specific fluorogenic substrate compared to PBS control (black line). Data are representative of three independent experiments, and statistical significance was determined by least squares ordinary fit. *$P < 0.5$. RFU, relative fluorescence units. (**F**) Ticagrelor (blue line) reduces intracellular calcium levels in human platelets loaded with 2 μM Fluo-3 dye and stimulated with recombinant α-toxin (5 μg/ml) compared to PBS control (black line); calcium influx was measured every 30 s by fluorescence. For both (E) and (F), α-toxin–stimulated platelets (whether ticagrelor- or PBS-treated) were normalized to their respective nonstimulated platelet controls. (**G**) Ticagrelor treatment of human platelets did not alter surface ADAM-10 expression as determined by flow cytometry. (**H**) Human platelets with or without ticagrelor treatment were infected with MRSA at MOI = 0.1 for 90 min. Surface Glycoprotein VI (GP6) was measured by flow cytometry, and percent decrease in expression (GP6 shedding) was calculated. (**I**) Human platelet P-selectin expression indicating platelet activation measured by flow cytometry with or without MRSA challenge (MOI = 0.1) and with or without ticagrelor for 90 min. All data are represented as mean ± SEM and are representative of at least three independent experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's multiple comparisons test (A, C, and G), unpaired two-tailed Student's *t* test (H), and two-way ANOVA with Bonferroni's multiple comparisons posttest (I). *$P < 0.05$, **$P < 0.005$.

micro-abscesses was identified within the renal glomeruli, tubules, and blood vessels of ticagrelor-treated mice versus phosphate-buffered saline (PBS) control animals, corroborating the CFU quantification data. Renal microabscesses in the control group were generally larger and more densely packed with bacteria, whereas those in ticagrelor-treated mice were frequently disrupted by immune cell infiltrates. Together, these data suggest that platelet P2Y12 inhibition blocks α-toxin– and SA-mediated platelet cytotoxicity and consequent thrombocytopenia, thus enhancing the platelet-mediated clearance of the pathogen in vitro and in vivo.

SA α-toxin activates endogenous platelet sialidase activity

Our clinical data and those of others (*26*), coupled with our experimental work and previous platelet depletion studies (*12*, *24*), suggest that platelet count per se is important in determining SA clinical outcome. Because α-toxin production correlated to thrombocytopenia in patients and in experimental mouse infection, we hypothesized that SA deploys the toxin as a means to deplete the host of an effective circulating innate immune cell. Yet, platelet senescence and clearance are tightly regulated by multiple mechanisms, in particular the highly conserved hepatic transmembrane heterodimeric AMR (*36*). The AMR clears "aging" platelets with reduced terminal α-2,3–linked sialic acids on their surface glycoproteins and glycolipids by engaging the exposed underlying galactose. We asked if the observed therapeutic effect of ticagrelor in SA sepsis was solely based on inhibiting platelet cytotoxicity or further intersected with this important mechanism of platelet homeostasis.

To assess platelet sialylation state during SA bacteremia, we obtained frozen plasma from 10 randomly selected adult patients with SA bacteremia, 10 patients with *Escherichia coli* bacteremia, and 5 healthy subjects. We found an increase in exposed galactose (indicative of desialylation) on the platelets of SA-infected patients compared to the two other groups (Fig. 4A). However, SA lacks a bacterial sialidase (neuraminidase) present in other pathogens including *S. pneumoniae* (Fig. 4B). Rather, we found that WT SA induced sialidase activity ($P < 0.005$) on purified human platelets, whereas its isogenic ΔHla mutant derivative did not (Fig. 4C). An increase in platelet sialidase activity in response to WT SA or purified α-toxin, present within the platelet pellet but not released into the media, was detected in independent assays using lectin affinity and a fluorescent substrate (fig. S8A and Fig. 4D). Although the precise mechanism of its transfer is not established, Neu1 is the main endogenous sialidase that can translocate from lysosomal stores to the platelet surface to target glycoproteins and expose AMR ligands (galactose) (*37*, *38*), and we confirmed its up-regulation in response to SA challenge by flow cytometry (fig. S8B). Probing the observed therapeutic effect of P2Y12 inhibition in this context, we found that ticagrelor strongly inhibited SA-induced platelet sialidase activity

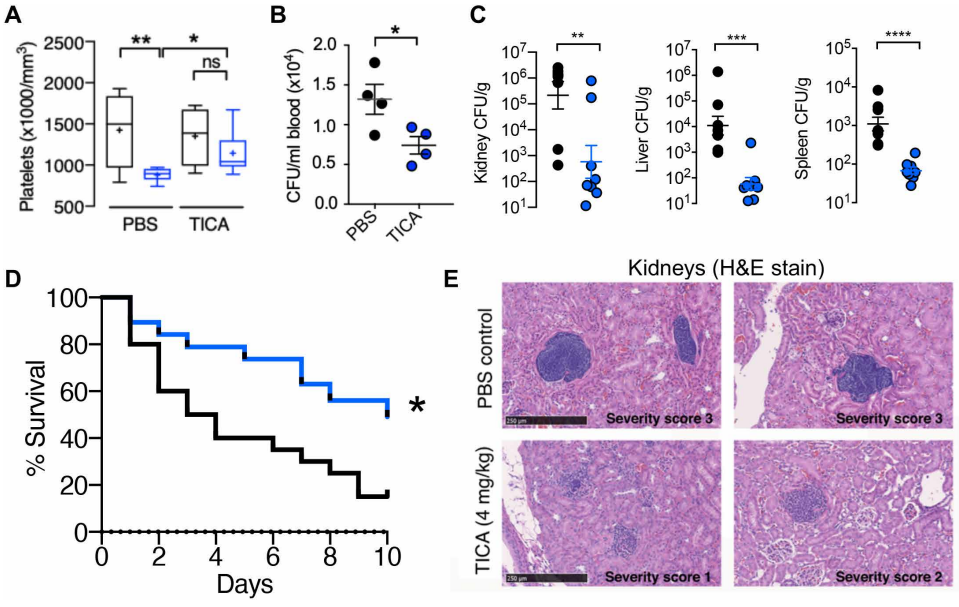

Fig. 3. FDA-approved P2Y12 inhibitor ticagrelor protects against SA bacteremia. (**A** and **B**) Outbred CD-1 mice treated with ticagrelor (4 mg/kg) or vehicle (1× PBS) by oral gavage every 12 hours for 72 hours and then challenged intravenously with 1×10^8 CFUs of SA; blood platelets and bacterial CFU burden were enumerated 4 hours after infection. (**C**) Enumeration of bacterial CFU burden at 72 hours in organs of mice pretreated with vehicle (PBS) or ticagrelor (4 mg/kg) 12 hours before intravenous SA and every 12 hours thereafter; ($n = 8$). (**D**) Mortality curves of outbred CD-1 mice pretreated with vehicle (PBS) or ticagrelor (4 mg/kg) beginning 24 hours before intravenous SA infection and then every 12 hours over a 10-day observation period ($n = 20$). Independent experiments were repeated twice, and data were pooled. (**E**) Hematoxylin and eosin (H&E) stain of representative histological kidney sections from mice pretreated with PBS vehicle or ticagrelor (4 mg/kg) 12 hours before SA infection and every 12 hours thereafter for 72 hours ($n = 8$). Yellow stars denote formation of dense bacterial colonies, and black arrows represent immune infiltrate. All histological sections are representative photos of at least six samples per two independent experiments. Where applicable, results are represented as mean ± SEM and statistical significance was determined by unpaired two-tailed Student's *t* test (B and C) and two-way ANOVA with Bonferroni's multiple comparisons posttest (A). For survival curves, statistical significance was determined by log-rank Mantel-Cox test (D); $^*P < 0.05$. For floating bar graphs, + denotes the mean, whiskers represent minimum to maximum, and floating box represents 25th to 75th percentile. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

(Fig. 4E). These results suggest that SA α-toxin–induced thrombocytopenia may not depend on wholescale platelet injury, but instead involve accelerated hepatic AMR-dependent clearance of desialylated platelets upon surface mobilization of Neu1. Binding of adenosine diphosphate (ADP) to P2Y12 elevates cytosolic calcium (Ca^{2+}) concentrations by stimulating phospholipase C–mediated production of inositol-1,4,5-trisphosphate (IP_3), thereby releasing Ca^{2+} from the intracellular stores through IP_3 receptor channels. Because Ca^{2+} is a major signaling molecule that allows for ADP-induced lysosomal secretion, P2Y12 inhibition in theory would block this process. However, a singular correlation between intracellular Ca^{2+} concentrations and granular secretion remains ambiguous, as our data, as well as older literature, suggest that there are both Ca^{2+}-dependent and Ca^{2+}-independent granular secretory pathways (*39*, *40*). That said, by linking sialidase activity to the ticagrelor therapeutic effect, additional target points for pharmacological support of platelet defense against SA came into view.

Inhibition of the hepatic AMR supports platelet-mediated defense against SA bacteremia

In previous work, we showed that moderate thrombocytopenia mediated by AMR-dependent clearance of desialylated platelets was protective in experimental sepsis caused by *S. pneumoniae*, a sialidase-expressing pathogen (*41*, *42*). However, as shown earlier in this study, *S. pneumoniae* is resistant to human platelet killing, and therefore, removal of desialylated and hypercoagulable platelets does not deplete the bloodstream of an effective antimicrobial effector cell type. We asked whether the innate immune calculus could prove different for platelet-sensitive SA by challenging WT and AMR-deficient ($Asgr2^{-/-}$) mice in the C57BL/6 background. Whereas WT mice remained highly sensitive to SA α-toxin–induced thrombocytopenia, platelet counts in $Asgr2^{-/-}$ mice did not drop after bacterial challenge (Fig. 5A), indicating that recruitment of AMR clearance was the main pathogenic driver of platelet clearance during infection. In contrast to findings in *S. pneumoniae* infection (*41*), $Asgr2^{-/-}$ mice, which are resistant to pathogen-induced thrombocytopenia, exhibited a strong survival advantage against lethal SA challenge (Fig. 5B). This genetic association could be reproduced pharmacologically in WT mice, where asialofetuin, a competitive glycoprotein inhibitor of the hepatic AMR (Fig. 5C), improved mouse survival in lethal SA challenge by maintaining platelet count during infection (Fig. 5D) and by reducing bacterial burden in the kidney, liver, and spleen (Fig. 5E). Corroborating that α-toxin–dependent desialylation drove the accelerated platelet clearance, no SA-induced reduction in platelet count was seen in mice lacking the hepatic AMR (Fig. 5, A and F).

FDA-approved sialidase inhibitor oseltamivir blocks AMR-mediated platelet clearance and protects against SA bacteremia

The above results showed that mice were protected against SA infection by ticagrelor, which inhibits α-toxin–induced platelet desialylation, or by genetic or pharmacological inactivation of the AMR, which blocks hepatic clearance of desialylated platelets. Further corroboration of the importance of platelet sialylation for maintaining bloodstream defense against SA bacteremia was obtained using mice lacking the *St3gal4* sialyltransferase gene, which show diminished platelet sialylation and baseline thrombocytopenia (*43*). Compared to WT C57BL/6 mice, the $St3gal4^{-/-}$ mice had accelerated mortality upon intravenous SA infection (Fig. 6A), but no further SA-induced reduction in their already low platelet counts (~25% of normal mice; Fig. 6B). Because sialidase (Neu1) activity appears central to the "toxin-platelet-AMR" pathway driving deleterious thrombocytopenia in SA bloodstream infection, we considered the possibility that pharmacological sialidase inhibition could be of therapeutic benefit. Oseltamivir (Tamiflu) is a commonly prescribed FDA-approved

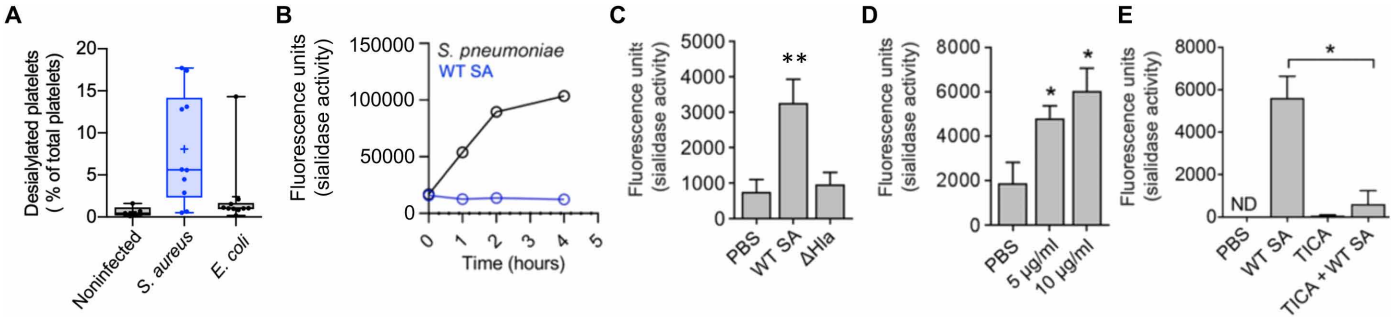

Fig. 4. SA α-toxin activates endogenous platelet sialidase activity. (**A**) Percent desialylated platelets in platelet-rich plasma from noninfected subjects or patients with SA or *E. coli* bacteremia measured by flow cytometry. (**B**) SA and *S. pneumoniae* sialidase activity assessed for over 4 hours. (**C**) Sialidase activity examined on washed human platelets exposed to WT SA or isogenic ΔHla for 1 hour or (**D**) sialidase activity examined on washed human platelets exposed to recombinant α-toxin (5 and 10 μg/ml) for 30 min. (**E**) Sialidase assay performed on washed human platelets treated with or without 10 μM ticagrelor and exposed to WT SA for 1 hour. Where applicable, all data represented as mean ± SEM and are representative of at least three independent experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's multiple comparisons test (A and C to E). *$P < 0.05$. ND, not detectable.

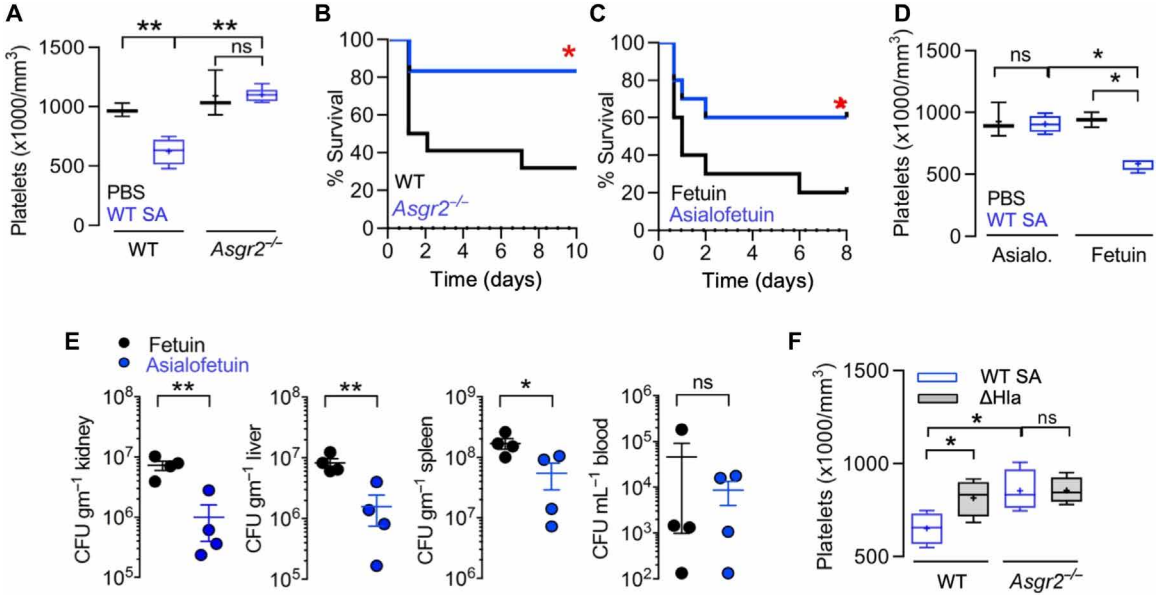

Fig. 5. Inhibition of the hepatic Ashwell-Morell receptor (AMR) supports platelet-mediated defense against SA bacteremia. (**A**) C57BL/6 WT ($n = 4$) and *Asgr2*$^{-/-}$ ($n = 6$) mice were challenged by intraperitoneal injection with SA, blood was harvested by cardiac puncture, and platelet count was enumerated. (**B**) Ten-day mortality study with WT ($n = 22$) and *Asgr2*$^{-/-}$ mice ($n = 16$) challenged by intraperitoneal injection with SA. Study was performed two independent times, and data were pooled. (**C**) Eight-day mortality study with WT treated with fetuin (n = 10) or asialofetuin ($n = 10$) and challenged by intraperitoneal injection with SA. (**D**) WT mice were treated with asialofetuin ($n = 4$) or fetuin ($n = 4$) and challenged by intraperitoneal injection with SA, platelet count was enumerated, and (**E**) kidneys, liver, spleen, and blood were harvested 24 hours after infection for bacterial CFU enumeration. (**F**) WT and *Asgr2*$^{-/-}$ mice challenged with WT MRSA or the isogenic ΔHla mutant. At 4 hours after infection, blood was harvested by cardiac puncture for enumeration of platelet count. Statistical significance was determined by unpaired two-tailed Student's *t* test (E), two-way ANOVA with Bonferroni's multiple comparisons posttest (A, D, and F), or log-rank (Mantel-Cox) test (B and C) for the survival curves. For floating bar graphs, + denotes the mean, whiskers represent minimum to maximum, and floating box represents 25th to 75th percentile. *$P < 0.05$, **$P < 0.005$.

drug designed to target influenza sialidase (neuraminidase) and lessen the severity of flu symptoms. However, oseltamivir has a degree of nonselectivity in its sialidase inhibition, as the drug was recently recognized to raise platelet counts in mice with anti-GPIbα–mediated thrombocytopenia (*44*). Using *Asgr2*$^{-/-}$ mice to prevent immediate clearance of desialylated platelets, we confirmed that oseltamivir inhibited platelet desialylation in vivo during SA infection ($P < 0.005$, Fig. 6C). Then, using WT mice, we showed that oseltamivir significantly reduced the degree of α-toxin–induced thrombocytopenia during WT SA infection ($P < 0.005$, Fig. 6D). Both oseltamivir and established human Neu1-selective sialidase inhibitor C9-butyl-amide-2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) significantly improved survival outcomes in lethal SA bacteremia ($P < 0.05$; Fig. 6E).

Last, to support all elements of the elucidated "toxin-platelet-AMR" pathway, we repeated several key in vivo experiments (the ticagrelor, oseltamivir, and asialofetuin treatment studies, as well as challenge of *Asgr2*$^{-/-}$ mice) with the SA ΔHla knockout mutant. Given the attenuated virulence of this mutant, establishment of bacteremia in the murine intravenous model required an eightfold

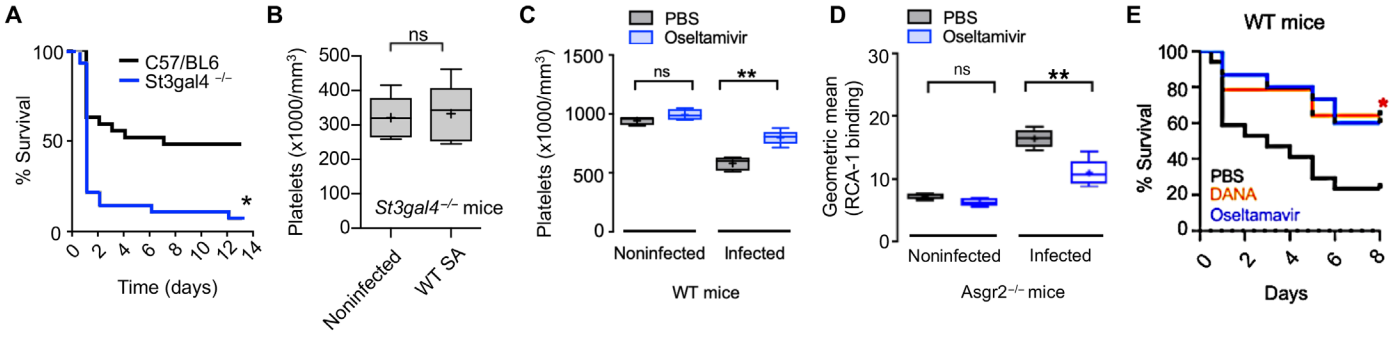

Fig. 6. FDA-approved sialidase inhibitor oseltamivir blocks AMR-mediated platelet clearance and protects against SA bacteremia. (**A**) *St3gal4*$^{-/-}$ mice that have decreased platelet sialylation and thrombocytopenia show accelerated mortality upon SA bloodstream infection ($n = 10$ per group). (**B**) circulating platelet count 4 hours after intravenous SA challenge in WT versus *St3gal4*$^{-/-}$ mice ($n = 10$ per group). (**C**) Platelets isolated from *Asgr2*$^{-/-}$ mice treated with or without oseltamavir and infected with MRSA were assessed for RCA-1 lectin binding. (**D**) WT C57BL/6 mice were treated with oseltamavir ($n = 6$) or PBS control ($n = 5$) and infected with WT SA by intraperitoneal injection. Blood was harvested 24 hours after infection, and platelet counts were collected. (**E**) Eight-day mortality study conducted on WT C57BL/6 mice treated with DANA ($n = 16$), oseltamavir ($n = 16$), or PBS control ($n = 16$). Statistical significance was determined by unpaired two-tailed Student's *t* test (B), two-way ANOVA with Bonferroni's multiple comparisons posttest (C and D), or log-rank (Mantel-Cox) test (A and E). For floating bar graphs, + denotes the mean, whiskers represent minimum to maximum, and floating box represents 25th to 75th percentile. Unless otherwise stated, *$P < 0.05$ and **$P < 0.005$.

higher challenge inoculum. Blood harvested 4 hours after infection in all the experiments showed that the platelet count drop associated with WT infection was not seen in ΔHla-infected mice in any of the models, even at the eightfold higher bacterial challenge inoculum (fig. S9A). Likewise, equal CFU counts of the ΔHla knockout mutant SA were recovered in the kidney, liver, spleen, and blood of control mice versus mice treated with either ticagrelor, asialofetuin, or oseltamivir, as well as WT mice when compared to *Asgr2*$^{-/-}$ animals (fig. S9B). As predicted by the model, deletion of α-toxin "phenocopies" the therapeutic benefits of the drug and genetic interventions to support platelet defense against SA bloodstream infection.

DISCUSSION

SA is a leading agent of human bloodstream infection, with higher morbidity and mortality rates than other common bacterial pathogens. Successful treatment of SA bacteremia remains vexing, as the pathogen deploys diverse mechanisms for resistance to immune and antibiotic clearance. Life-threatening complications of SA bacteremia such as metastatic infections, infective endocarditis, and disseminated intravascular coagulation drive worsened patient outcomes. Thrombocytopenia (platelet count <150,000/mm^3 blood) is a common phenotype observed during bacteremia and is the most predictive independent risk factor for bacteremia-associated mortality, especially in cases of neonatal sepsis and critically ill septic patients in the ICU (*45*, *46*). Although the underlying cause of thrombocytopenia is multifactorial, our mechanistic analysis of platelet-mediated defense establishes a central pathophysiologic framework aggravating platelet depletion during SA bacteremia. We show that for SA to evade platelet microbicidal activity, the pathogen deploys the cytotoxic α-toxin, which injures platelets and stimulates the release of endogenous sialidase, thereby dysregulating the platelet clearance mechanism involving the hepatic AMR. Pharmacological targeting of multiple levels of this "toxin-platelet-AMR" pathway revealed new strategies to mitigate the progression of this immunocompromised state and protect against lethal SA bacteremia (fig. S10).

Sialidase transfer from lysosomal compartments to the platelet cell surface may potentially be elicited by multiple surface-bound receptors including but not limited to P2Y12, PAR-1, and PAR-4 (*47*). These receptors have converging intracellular signaling pathways, and studies indicate that P2Y12 functions in cross-talk with PAR receptors (*48*). We find here that ticagrelor, a commonly prescribed FDA-approved P2Y12 inhibitor, hastened clearance of SA bacteremia in vivo and enhanced human platelet killing of SA ex vivo. Here, we describe effects of the drug in reducing SA α-toxin–mediated platelet cytotoxicity, inhibiting activation of endogenous platelet sialidase activity, and preventing AMR-dependent platelet clearance. As thrombocytopenia was not observed in SA-challenged mice lacking the AMR, ticagrelor's ability to counteract wholescale SA-induced platelet damage may be unique to the high bacterial concentrations and close platelet contact present in our in vitro assays, and perhaps only relevant in vivo within an infected thrombus. Conceivably, ticagrelor's primary therapeutic indication for acute coronary syndrome, reduction of platelet aggregation, may further provide protective benefit in SA bacteremia as the pathogen produces two clotting factors, coagulase (Coa) and von Willebrand factor binding protein (vWbp), that contribute to abscess formation and systemic virulence (*49*). One previous report linked in vitro P2Y12 activation to the release of platelet antimicrobial peptides active against SA (*50*). This paradoxical result may, in part, reflect the particular SA strain (ISP479C) used in the study, which harbors a chromosomal Tn551 insertion with a pleiotropic effect on several extracellular and cell wall proteins, including elimination of measurable α-toxin activity (*51*). Platelet release of antimicrobial peptides active against SA is also activated by additional pathways, including thrombin-mediated enzymatic activation of cell surface PAR-1 still operative during P2Y12 blockade (*12*). The feasibility of ticagrelor as an adjunctive therapy for SA bacteremia in complex ICU patients is likely enhanced by its reversible binding to the P2Y12 receptor binding, providing a very rapid onset and offset of action (*52*).

Oseltamivir, a commonly used FDA-approved influenza sialidase inhibitor, maintained platelet sialylation to delay AMR-dependent platelet clearance and thus provided protection against mortality in

SA bloodstream infection. Although one enzymatic study suggested that oseltamivir had only limited inhibitory activity against human sialidases (*53*), humans prescribed oseltamivir show higher platelet counts than matched controls [independent of proven influenza; (*54*)], and two independent case studies report successful use of the drug to restore platelet counts in a patient with immune thrombocytopenia (*55*, *56*). Bacterial coinfection is estimated to have contributed to nearly all influenza deaths in the 1918 influenza pandemic and up to one-third of 2009 pandemic influenza A (H1N1) infections managed in ICUs worldwide (*57*). In particular, the potential for lethal synergism between SA and influenza virus has recently been documented in U.S. clinical epidemiologic studies of adult and pediatric patients (*58*, *59*). In laboratory-confirmed influenza, an inverse relationship between virus load and platelet count is seen, and viral-induced thrombocytopenia can be recapitulated in the ferret model (*60*). We speculate that the "two-hit" scenario of influenza neuraminidase on top of α-toxin–induced endogenous sialidase activation may accelerate platelet clearance, depleting the host of a critical frontline defense against SA bloodstream dissemination, thus increasing the odds of fatal outcome.

An important limitation of pharmacologically targeting AMR-dependent clearance of desialylated platelets to treat bacteremia is its dependency on sensitivity of the offending pathogen to platelet antimicrobial activity. A definitive or strong presumptive microbiologic diagnosis of SA would be required, precluding its use as empiric therapy wherein other pathogens such as platelet-resistant *S. pneumoniae* could yield adverse results (*42*). Multiple pathogenic mechanisms contribute to sepsis, and intrinsic host factors can have differing roles depending on the pathogen involved (*61*). In this case, AMR function may serve protective and disadvantageous roles depending upon the pathogen and the balance of platelet action in thrombosis versus antimicrobial activity. Previous research indicates that loss of AMR can increase platelet count and regulate thrombopoietin production (*62*). Mechanisms of physiologic platelet turnover remain to be fully established and are likely to contribute to therapeutic modulation in the future. However, it is unlikely that either ticagrelor or oseltamivir administered late in the course of severe SA-induced thrombocytopenia could quickly restore platelet counts. There, perhaps platelet transfusion could augment anti-SA killing capacity in blood, wherein the pharmacological agents could mitigate against further α-toxin–driven accelerated desialylation and AMR clearance of the donor platelets. The pathological process can also be targeted upstream at the level of the inciting SA α-toxin, where important research studies on neutralizing antibodies (for example, Medimmune 4893) and receptor antagonists (for example, GI254023X) have shown promising results (*14*, *63*).

Therapeutic drug repurposing is an important avenue of exploration to improve clinical outcomes in serious infections where high rates of treatment failure and antibiotic resistance jeopardize patients. Elucidation of sialidase-dependent platelet homeostasis as a key battleground in host defense against SA bloodstream infection revealed the potential utility of P2Y12 and sialidase inhibition as adjunctive agents to antibiotic treatment and ICU supportive care for the critically ill. The most effective physiological concentrations to inhibit platelet cytotoxicity and sialidase activity and protect against SA bacteremia in humans are currently unknown. As FDA-approved drugs with excellent safety profiles in each class are readily at hand, we hope that carefully designed clinical investigation to validate or refute our experimental observations may follow.

MATERIALS AND METHODS

Study design

The objective of this study was to understand the mechanistic basis of platelet homeostasis and function during SA bacteremia to guide future therapeutic approaches. We analyzed patient data and SA isolates from a published 2009–2010 institutional review board (IRB)–approved study (*25*) of SA bacteremia at the University of Wisconsin Hospital (a 493-bed academic medical center in Madison, WI) to link thrombocytopenia to patient mortality and elevated α-toxin production. We corroborated both associations in a University of California (UC) San Diego Institutional Animal Care and Use Committee (IACUC)–approved murine model of SA bacteremia. Additional UC San Diego IRB-approved ex vivo studies with freshly isolated human platelets found that the FDA-approved P2Y12 antagonist ticagrelor blocked α-toxin–induced platelet injury and sialidase activation, improving microbial killing. Infection studies in WT and isogenic AMR-deficient mice were used to link α-toxin–mediated platelet sialidase activation to accelerated thrombocytopenia and impaired SA clearance, which was counteracted by ticagrelor or the FDA-approved sialidase inhibitor oseltamivir.

Ethics statement

Animal studies were conducted in accord with protocols approved by the UC San Diego IACUC; all efforts were made to minimize animal numbers and suffering. Blood for platelet isolation was obtained via venipuncture from healthy volunteers under written informed consent approved by the UC San Diego Human Research Protection Program.

S. aureus patient isolates

Consecutive patients from the above previously published, IRB-approved study (*25*) and its ongoing continuation (IRB #2018-0098) with blood cultures of SA from April 2009 to March 2010 at the University of Wisconsin Hospital were analyzed for α-toxin expression by Western immunoblot and densitometry band analysis by ImageJ. Levels of α-toxin expression were grouped in the following order: low: >10,000; medium: 10,000 to 20,000; high: >20,000. Patient demographics, blood work (including platelet and leukocyte counts), and infection source were collected at time of administration. Bacterial isolates were obtained at the onset of presentation and stored at −80°C until analysis. All laboratory tests were performed by investigators blinded to patient information.

Asgr2$^{-/-}$, *St3gal4*$^{-/-}$, and AMR inhibitor mouse infection studies

Eight- to 12-week-old *Asgr2*$^{-/-}$ mice (*43*) or 10- to 14-week-old *St3gal4*$^{-/-}$ mice on a C57BL/6 (The Jackson Laboratory) genetic background and WT mice bred and raised in the same room were used. WT SA was grown overnight [shaking at 37°C in Todd-Hewitt broth (THB)] and washed once in 1× PBS, 1×10^8 CFUs were injected intraperitoneally unless otherwise specified in the figure legend, and mortality was observed over the course of 10 days. For AMR inhibitor studies, WT C57BL/6 mice were treated with asialofetuin or fetuin (25 mg/ml) before intraperitoneal challenge with 1×10^8 CFU SA; mortality was observed over the course of 8 days. For both studies, mice that appeared moribund were euthanized by CO_2 asphyxiation. Platelet count enumerations were performed 4 hours after infection. For AMR inhibitor CFU enumeration, mice were euthanized 24 hours after infection, organs were harvested, and dilution was plated onto Todd-Hewitt agar (THA).

Sialidase inhibitor mouse infection studies

Eight- to 10-week-old WT C57BL/6 mice were treated with oseltamivir (5 mg/kg) in 100 μl of PBS or the Neu1-selective inhibitor DANA (2 mg/kg) at the time of infection and 3 hours after intraperitoneal infection with 1×10^8 CFU SA. Platelets were enumerated and sialidase activity was assessed using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU; Sigma Aldrich) from blood collected 4 hours after infection using a previously described protocol (*4*). For ex vivo sialidase analysis, murine platelet-rich plasma (PRP) was isolated by cardiac puncture with a 25-gauge needle attached to a syringe containing 100 μl of Anticoagulant Citrate Dextrose Solution (ACD-A) and centrifuged at 100*g* for 10 min without braking. After isolation, 25 μl of PRP was added to wells of white 96-well plate (Costar) with 25 μl of RPMI + 125 μM 4MU. The plate was incubated at 37°C + 5% CO_2 for 30 min, followed by an addition of 1 M Na_2CO_3, and fluorescence was determined at excitation 530 nm and emission 585 nm.

Ticagrelor treatment mouse infection studies

SA was grown (shaking overnight at 37°C in THB) and washed once in PBS, and 1×10^8 CFUs were injected intravenously into outbred 8- to 10-week-old CD1 (Charles River Laboratories) mice. Where indicated, ticagrelor (4 mg/kg) or vehicle (1× PBS) was delivered by oral gavage 24 hours before and every 24 hours after infection over a course of 10 days. Mice that appeared moribund were euthanized by CO_2 asphyxiation. For quantification of CFU burden and histological preparation, mice were treated with ticagrelor (4 mg/kg) or vehicle (1× PBS) 12 hours before and every 24 hours after intravenous injection of 1×10^8 CFU SA. At 12 hours after infection, two mice from each group were euthanized by CO_2 asphyxiation and the kidneys, spleen, heart, and liver were harvested and fixed in 10% neutral-buffered formalin for 24 hours, then routinely processed, and paraffin-embedded for histological analysis. Five-micrometer-thick hematoxylin and eosin–stained sections of each tissue were examined by a veterinary pathologist blinded to the treatment group. Distinct bacterial colonies visible at ×4 magnification were counted in three longitudinal sections of heart and six longitudinal sections of kidney. Lesions related to bacterial infection were described and graded (minimal, 1; mild, 2; moderate, 3; or severe, 4) based on degree of tissue damage. At 72 hours after infection, the remaining surviving mice were euthanized by CO_2 asphyxiation, blood was collected by cardiac puncture, and organs were excised. Blood and organ homogenate (MagNA Lyser instrument, Roche Diagnostics Corporation) were serially diluted in molecular grade H_2O and plated onto THA for bacterial CFU enumeration. The study was performed three independent times, and data from a representative experiment were shown. For platelet quantification, mice were treated with ticagrelor (4 mg/kg) or vehicle (1× PBS) every 12 hours for 72 hours before intravenous injection of 1×10^8 CFU SA. Four hours after infection, blood was collected by cardiac puncture with a 25-gauge needle attached to a syringe containing 100 ml of ACD buffer and transferred into EDTA tubes and a complete blood count was obtained.

Statistical analysis

All in vitro, ex vivo, and in vivo data were collected from three or more independent experiments with three or more biological replicates and are represented as mean ± SEM, unless otherwise stated. For descriptive data (transmission electron microscopy and histopathologic staining), experiments were performed at least twice independently with three or more biological replicates and illustrated as best representative images. The α level used for all tests was 0.05; the data were normalized (single outliers removed via Grubbs' test if applicable) and unpaired Student's *t* test, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test, or two-way ANOVA with Bonferroni's multiple comparisons test was performed as explained in figure legends to determine statistical significance. For comparison of survival curves, a log-rank (Mantel-Cox) test was performed. Statistical analyses were done using GraphPad Prism version 8.42 (GraphPad Software Inc.). *P* values <0.05 were considered statistically significant.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Thrombocytopenia established by anti-CD41 antibody treatment of mice.

Fig. S2. Grouping of α-toxin expression of SA isolates from bacteremia patients.

Fig. S3. Generation of an isogenic SA ΔHla mutant.

Fig. S4. Exclusion of ticagrelor off-target effects on SA immune cell interactions and growth.

Fig. S5. Additional effects of SA or ticagrelor on human platelet phenotypes in vitro.

Fig. S6. Effects of SA challenge on platelets and thrombopoiesis in vivo.

Fig. S7. Organ pathology in murine SA infection with or without ticagrelor treatment.

Fig. S8. Neu1 is predominantly detected on the platelet cell surface and is induced by SA exposure.

Fig. S9. SA α-toxin deletion mutant phenocopies protective effects of ticagrelor, oseltamivir, and AMR loss or inhibition.

Fig. S10. Schematic illustration of proposed "toxin-platelet-AMR" pathway exploited by SA in the pathogenesis of bloodstream infection.

Data file S1. Primary values for main and supplemental figures (Excel).

References (*64*–*66*)

View/request a protocol for this paper from *Bio-protocol*.

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Repurposed drugs block toxin-driven platelet clearance by the hepatic Ashwell-Morell receptor to clear *Staphylococcus aureus* bacteremia

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Promoting platelet persistence

Blood infection by *Staphylococcus aureus* (SA) can be fatal. Sun *et al.* found that lowered platelet counts associated with mortality in a cohort of patients with bacteremia, and pinpointed SA-induced desialylation and hepatic clearance of platelets as the cause. This reduction in circulating platelets was counteracted by either of two approved drugs, the sialidase inhibitor oseltamivir or ticagrelor, an inhibitor of platelet receptor P2Y12, leading to improved outcomes in a mouse model of SA-induced bacteremia. This study supports the possibility of repurposing two clinically prescribed drugs against bloodstream infection by SA.