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Supplementary Materials for

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

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/13/586/eabd6737/DC1)

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

MATERIALS AND METHODS

Measurement of desialylation of patient platelets

In the above patient biobank, 2-5 mL of patient whole blood is collected on the day of presentation with bacteremia at the hospital. In this analysis, platelets from patients with *Staphylococcus aureus* (SA) bacteremia were compared to patients with *Escherichia coli* bacteremia and sepsis, and uninfected healthy volunteers. All patients with SA bacteremia were diagnosed from an endovascular source by an infectious diseases physician. Patients with *E. coli* bacteremia/sepsis had infection sources from the urinary or gastrointestinal tract. No patients received P2Y12 antagonists or any other anti-platelet therapy at the time of collection. Samples were processed to separate plasma and cells, which are then aliquoted separately stored at -80°C until analysis. Platelets in plasma from patients admitted with SA, *Escherichia coli* sepsis, or normal controls were analyzed for RCA-1 binding by flow cytometry. Plasma samples were stained with PerCP/Cy5.5 anti-human CD41 antibody (Biolegend) and Fluorescein labeled Ricinus Communis Agglutinin I (RCA-1; Vector Laboratories). % desialylated platelets were calculated using the Flojo software.

Bacterial strains and plasmids

Community-acquired methicillin-resistant SA (MRSA) strain USA300 (TCH1516) and its isogenic ΔHla mutant lacking α-toxin, were used in the study. Targeted mutagenesis of *Hla* was conducted by precise, markerless allelic replacement of USA300 TCH1516 *hla* gene (Locus tag USA300HOU_1099, NC_010079.1 (1170314.1171273, complement) by PCR-based methods adapting the pKOR1 knock-out strategy previously described for SA mutagenesis *(64)*. As shown in fig. S3, sequence immediately upstream of *hla* was amplified with the primers D and E and that immediately downstream of *hla* with the primers B and F. Primers B and D were constructed with ~25 bp 5’ overhangs for the opposite flanking

region. Upstream and downstream PCR products were fused using primers E and F in a second round of PCR. The amplicons were then subcloned into temperature-sensitive vector pKOR1 using the BP clonase reaction (Invitrogen). The resulting plasmid pKOR1-hla was passed through SA RN4220, and the purified plasmid electroporated into strain TCH1516. Precise in-frame allelic replacement of *hla* was established by a two-step process of temperature shifting and anti-sense counterselection and confirmed by PCR. Other listed primers (**fig. S3**) were used for PCR confirmations and for screening potential mutants and clones. All strains were routinely grown in Todd Hewitt broth (THB) and propagated shaking at 37°C to mid-log phase (optical density 600 nm (OD_{600}) = 0.4) unless otherwise stated. Bacteria were collected by centrifugation at 4,000 RPM x 10 min, washed once in phosphate-buffered saline (PBS), and resuspended to the desired dilution in PBS.

Platelet and neutrophil isolation

For platelet isolation, human venous blood was drawn using a 20G needle from healthy human donors using acid-citrate-dextrose buffer (ACD; Sigma) as an anticoagulant (1:6 v/v), unless otherwise stated. To obtain platelet-rich plasma (PRP), blood was centrifuged at 1,000 RPM x 10 min with no brake. To avoid contaminations with other cell types, only the upper two thirds of the platelet-rich plasma fractions were used. PRP was centrifuged at 1.500 RPM x 10 min. Isolated platelets were resuspended in serum-free, antibiotic-free, inhibitor-free RPMI (without phenol red) at room temperature. Blood was drawn according to a protocol approved by the local ethics committee. For neutrophil isolation, venous blood was drawn from healthy donors as above but using heparin as an anticoagulant. Purified neutrophils were collected using Polymorph Prep (Axis-Shield, Dundee, Scotland) per manufacturer's protocol.

Platelet cytotoxicity

Human platelets were pre-treated with 10 μM ticagrelor (Sigma) or vehicle control and incubated with rotation at 37° C + 5% CO_2 for 20 min, then exposed to 5 μg/mL recombinant α-toxin (H9395 Sigma**)** for an additional 30 min. Samples were spun down at 500 x *g* for 5 min, and supernatants evaluated using a commercial lactate dehydrogenase (LDH) assay (Promega) or ATP assay (Promega, CellTiter-Glo® Luminescent Cell Viability Assay).

Human platelet sialidase (neuraminidase) assay

Washed human platelets (3 x 10^7) were assayed for sialidase activity using a slight modification of the previously published 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU; Sigma) assay *(37, 65)*. Platelets were added to a white 96-well plate (Costar), pretreated with or without the identified drugs for 15 min, and exposed to 3 x 10^7 CFU of SA (MOI = 1:1) for 1 h at 37°C in 5% CO_2. Next, 125 μM 4MU was added and incubated 30 min at 37ºC before 1M Na_2CO_3 was added to each sample and fluorescence determined at excitation 530 nm and emission 585 nm. Background fluorescence was measured following the same procedure but in the presence of 1 mM sialidase inhibitor DANA.

Human platelet ADAM10 protease assay

Human platelets were isolated from healthy donors using hirudin as an anticoagulant. Isolated platelets were resuspended in serum-free, antibiotic-free, inhibitor-free RPMI (without phenol red) at room temperature. Human platelets (2×10^7) were pre-treated with 10μM ticagrelor (Sigma) or vehicle control and incubated at 37ºC with rotation for 20 min, at which time platelets were exposed to 5 mg/mL recombinant α-toxin and a fluorogenic ADAM10 specific substrate (PEPMCA001, Biozyme) at 37ºC + 5% CO2 without shaking. Fluorescence was measured every 15 min for 2.5 h using wavelengths of 325 nm (excitation) and 393 nm (emission).

Calcium assay (Fluo-3 assay)

Human donor blood was collected using hirudin as an anticoagulant. Platelet-rich plasma (PRP) was isolated and incubated with 2mM Fluo-3 AM (Thermo Fisher Scientific) at 37ºC + 5% CO2 with rotation for 20 min. Isolated platelets were resuspended in serum-free, antibiotic-free, inhibitor-free RPMI (without phenol red) at room temperature, pre-treated with 10μM Ticagrelor (Sigma) or vehicle control for 15 min, then exposed to 5 μg/mL recombinant α-toxin. Fluorescence was measured at 505 nm excitation and 530 nm emission every 30 sec.

Transmission electron microscopy

Isolated human platelets were pre-treated with 10 μM ticagrelor (Sigma) or vehicle control at 37°C + 5% CO_2 for 20 min, then infected with MRSA at MOI = 0.01 for an additional 2 h. Samples were fixed in modified Karnovsky's fixative (2.5% glutaraldehyde + 2% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.4) for at least 4 h, post-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 h, and stained in block in 2% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50–60 nm on a Leica UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken with an Eagle 4k HS digital camera (FEI). Images were taken from multiple random fields at 1200 ×, 2900 ×, 23,000 ×; gross morphology was analyzed in a blinded fashion.

Bacterial growth curves

Sterile non-pyrogenic tubes containing THB treated with ticagrelor (10 μM) or untreated were inoculated with overnight bacterial cultures to achieve an optical density (600 nm) of 0.1. Tubes were incubated in a shaking 37°C incubator, and absorbance measured every 30 min (600nm) for 8 h using a Spectronic 20D+ spectrophotometer (Thermo Scientific, Waltham, MA, USA)

Bactericidal assays

Human platelet killing: Isolated platelets were pre-treated with 10μM ticagrelor (Sigma) or vehicle control for 20 min at 37°C + 5% CO for 20 min, then infected with MRSA at MOI = 0.01 for 2 h. Infected platelets were sonicated (Fisher Sonic Dismembrator 550) for 3 sec, serially diluted, and plated THA plates. Percent killing by platelets as surviving CFU vs. original inoculum. *Human neutrophil killing:* Freshly isolated human neutrophils (PolymorphPrep) in serum-free RPMI were added to 96-well plates at 5×10^4 cells per well and treated with ticagrelor (10 μM) or vehicle control for 1 h at 37 °C + 5% CO_2, were infected with MRSA at MOI = 0.01 for 2 h. After incubation, cells were lysed with 0.025% Triton X-100, serial diluted, and plated on THA for CFU enumeration and determination of percent killing vs. inoculum. *THP-1 macrophage killing:* The THP-1 monocyte cell line authenticated by ATCC were cultured in RPMI (with phenol red) medium + 10% fetal bovine serum (FBS). Cells were differentiated in a 96 well format for 48 h with 25 nM phorbol myristate acetate (PMA, Sigma) with a subsequent 24 h rest period in RPMI + 10% FBS. On the day of infection, cells were washed once with PBS, treated with ticagrelor (10 μM) or untreated control for 1 h, and infected with MRSA at MOI 1:100. After incubation, cells were lysed with 0.025% triton X-100, serial diluted, and plated on THA for CFU enumeration and determination of percent killing vs. inoculum. *Human whole blood killing:* Whole blood was drawn from healthy donors using anticoagulant citrate dextrose solution (ACD, 1:6). Blood was pre-treated with varying concentrations of ticagrelor or vehicle control at 37°C + 5% CO_2 with rotation, then infected with MRSA (1:10) and incubated for an additional 1 h. After incubation, samples were sonicated (Fisher Sonic Dismembrator 550) twice at 20% maximum power for 3 sec with 10 seconds interval, serially diluted, and plated on THA for CFU enumeration and determination of percent killing vs. inoculum.

Induction and quantification of neutrophil extracellular traps

To induce extracellular trap production, neutrophils were seeded in 96-well plates at a density of 5 x 10^4 cells per well in RPMI (without phenol red). Cells were incubated with ticagrelor (10 uM) or untreated

control at 37°C with 5% CO_2 for 1 h before addition of NET-inducing PMA (25nM) for an additional. Extracellular DNA content was quantified using a Quant-IT PicoGreen dsDNA Assay Iit (Life Technologies, Carlsbad, CA) per manufacturer's instructions.

Human platelet surface P-selectin, ADAM-10, GP6 and CD63 measurements

The surface expression of P-selectin, ADAM-10, GP6 and CD63 were measured by flow cytometry. Human platelets were first incubated with TICA or vehicle control for 20 min in 37°C. After a wash with PBS, 1 x 10^7 platelets were incubated with SA, α-toxin or control media for 90 min. In each tube, antibodies against P-selectin, ADAM-10, GP6 or CD63 each conjugated with PE (Biolegend) were added. Expression of each molecule on the platelet surface was detected using FACSCalibur (BD) and analyzed using Flo Jo software (Flojo LLC).

Human platelet β-galactosidase activity

Human platelets were incubated with RPMI only, SA or SA and ticagrelor. After 90 min incubation at 37ºC, the samples were centrifuged at 2000 rpm for 10 min to collect supernatants. β-galactosidase activity in platelet supernatants was measured using the Mammalian β-Galactosidase Assay (Thermo-Fisher) per the manufacturer's protocol.

Mouse infection and platelet count determination

Wild-type SA and isogenic ΔHla mutant cultures were grown shaking overnight at 37°C in THB, washed once in 1x PBS, and 1 x 10^8 colony forming units (CFU) were injected intravenously (i.v.) into outbred 8- to 10-week-old CD1 mice (Charles Rivers Laboratories). Blood was collected, platelet count determined, and samples serial diluted and plated onto Todd Hewitt agar (THA) plates for CFU enumeration. Platelet count and CFU burden were enumerated 4 h post infection. For platelet depletion studies, endotoxin and azide-free 1 mg/kg anti-CD41 antibody (clone MWReg30, Biolegend) or 1 mg/kg isotype control rat IgG1

(clone RTK2071, Biolegend) were injected intraperitoneally (i.p.). 1 x 10^8 SA was administered by tail vein injection (i.v.) 16 h post-antibody treatment, and 4 h later mice were euthanized by CO_2 inhalation, blood collected by cardiac puncture with a 25G needle attached to a syringe containing 100 mL ACD buffer, and a complete blood count (CBC) was obtained. Blood, liver, spleen and kidneys were harvested, homogenized, and plated in serial dilutions onto THA plates for CFU counts. For *ex vivo* platelet depletion analysis, mice were treated as above and blood collected by cardiac puncture 16 h after antibody injection, and 1 x 10^6 SA added to the platelet-depleted and control blood and incubated at 37°C with rotation for 1 h prior to dilution plating for CFU counts. For platelet count determination in a time course after infection, 5 x 10^7 SA were administered i.v. a 50 µl blood collected by submandibular bleeding into EDTA tubes at each time point. Platelet counts were analysed using ProCyte Dx Hematology Analyzer (IDEXX).

Measurement of GP6 expression on murine platelets

GP6 expression on murine platelets during SA systemic infection was measured by flow cytometry. Blood collected above for time course platelet count was incubated with anti-mouse GP6 antibody (Emfret). Expression of GP6 and percentage GP6 negative platelets were analyzed using Flo Jo software (Flojo LLC).

Analysis of bone marrow megakaryocyte ploidy in murine SA systemic infection

Measurement of megakaryocyte counts in bone marrow and evaluation of megakaryocyte ploidy was performed as described previously *(66)*. In short, BM cells were harvested from femurs of mice 72 h after SA (5 x 10^7 CFU) i.v. infection (n = 3 in each group). After gently suspending the BM cells in 0.5% BSA PBS with 2 mM EDTA, cells were fixed in 2% paraformaldehyde at 4°C for 2 h, then washed and resuspended in PBS/EDTA with Fc Block for 10 min. Next 1 µg/mL Brilliant Violet 421™ anti-mouse CD41 antibody (Biolegend), 75 µg/mL propidium iodide (Sigma-Aldrich) and 45 µg/mL RNAse A (Qiagen) were added and incubated for 30 min. Equal volumes of each sample (100 µl) were analyzed by

flow cytometry using a NovoCyte (ACEA Biosciences). Megakaryocytes were identified as CD41+ cells and megakaryocyte number/ploidy was analyzed for 4 min.

Platelet microparticle analysis by flow cytometry

Isolated human platelets were infected with SA strains for various time points. After each time point, samples were collected, centrifuged and the supernatant analyzed using flow cytometry for microparticle content by adding FITC-conjugated anti-CD41 antibody (Biolegend). FITC positive particles smaller than 1 µm latex beads were selected. The absolute count was calculated from the 15 µm beads added to the same samples. For counting platelet microparticles in mouse serum, animals were infected with 1 x 10^8 SA i.v., blood collected 4 h after infection, and the serum separated. After adding FITC conjugated anti-CD41 antibody, microparticles in serum were analyzed by FACS as described above.

Mouse infection studies with the SA Δ*hla* mutant

Mice treated with ticagrelor, oseltamivir, asialofetuin or *Asgr2*$^{-/-}$ mice were infected with 8 x 10^8 SA ΔHla mutant strain i.v. and platelet count and bacterial burden analyzed as described above.

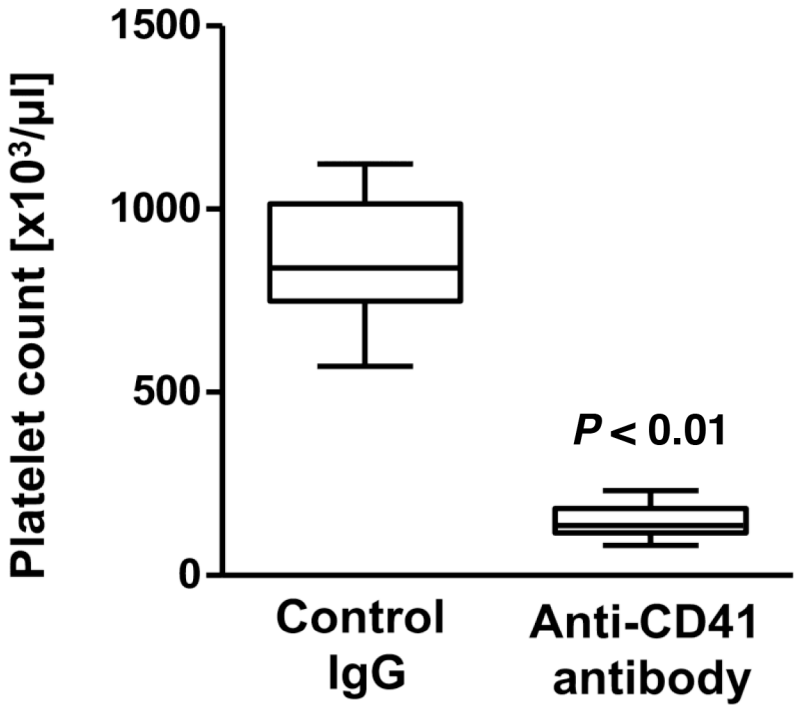


Fig. S1. Thrombocytopenia established by anti-CD41 antibody treatment of mice. Endotoxin and azide-free 1 mg/kg anti-CD41 antibody (clone MWReg30, Biolegend) or 1 mg/kg isotype control rat IgG1 (clone RTK2071, Biolegend) were injected intraperitoneally (i.p.) and blood collected by for platelet enumeration 16 h post-antibody treatment.

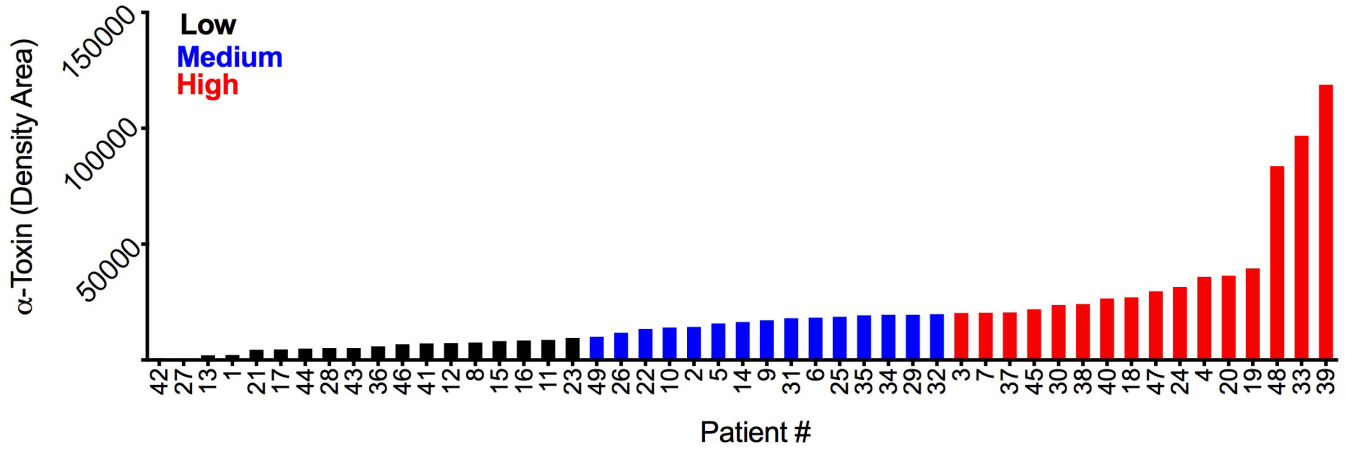
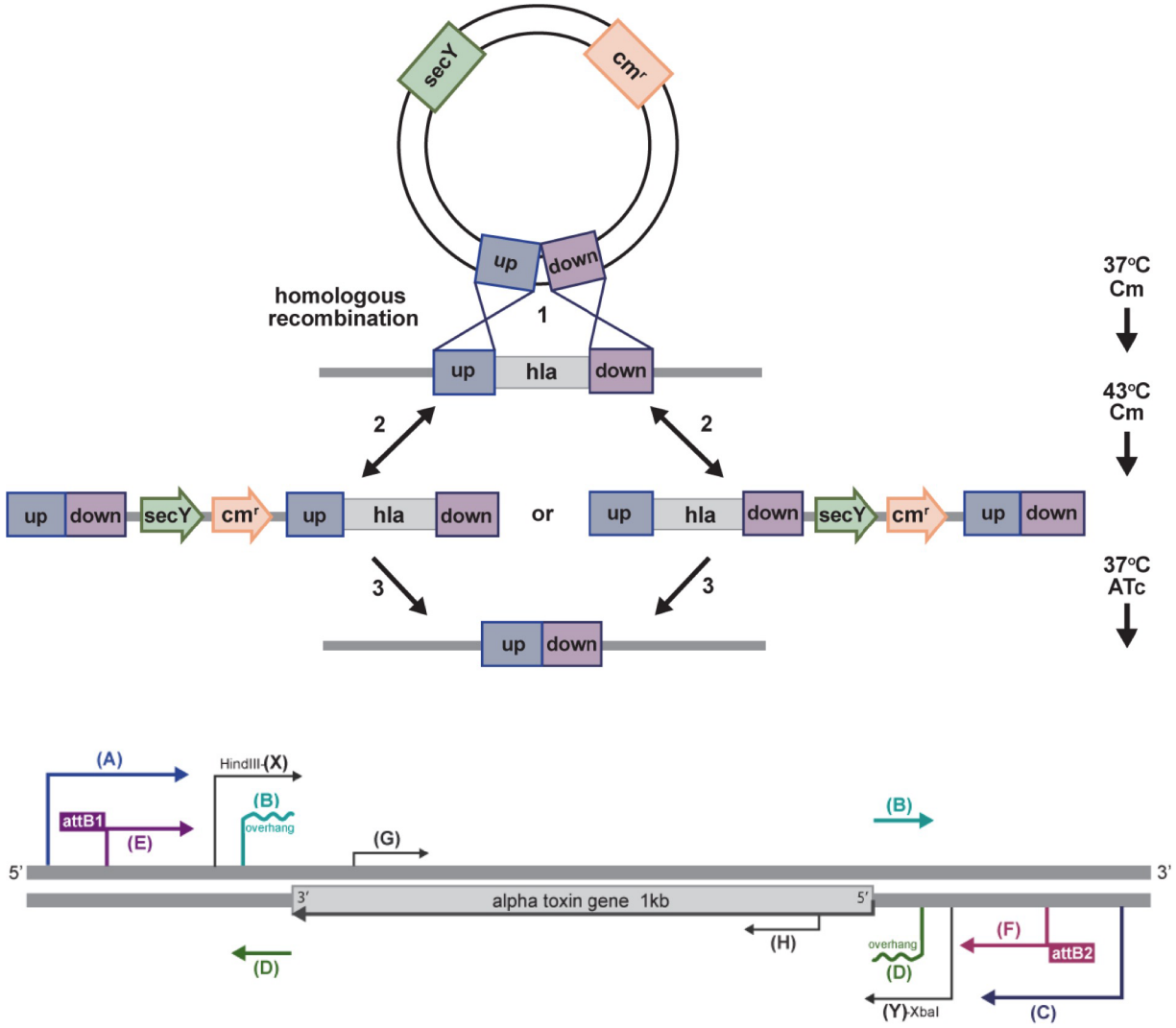


Fig. S2. Grouping of α-toxin expression of SA isolates from bacteremia patients. Western immunoblot band analysis on 49 patient clinical isolates grouped according to density area. Groups: low <10,000 (*black*); med. <20,000 (*blue*); high >20,000 (*red*). Densitometry performed using ImageJ software. Data represented as mean only.



Primers

A	**UpConstructUp**	**5'-gatgaagaagtccatacaaaatccgcatcattg-3'**
B	**DownConstructUp**	**5'-catgaacaaataatttacatttcatcatccttctatttttttaaaac-3'**
C	**DownConstructDown**	**5'-catgggcattttcattcggtcaactactaccaatataatattgtagagcctaag-3'**
D	**UpConstructDown**	**5'-aatagaaggatgatgaaatgtaaattatttgttcatgtca-3'**
E	**attB1 + Up**	**5'-ggggacaagtttgtacaaaaaagcaggctgaaattaatcatgcatg-3'**
F	**Down + attB2**	**5'-ggggaccactttgtacaagaaagctgggttattccttcatttaatg-3'**
G	**Confirm F**	**5'-ccaatttgttgaagtccagtgcaattgg-3'**
H	**Confirm R**	**5'-cctgtcgtcaatgccgcagattct-3'**

Fig. S3. Generation of an isogenic SA ΔHla mutant. Scheme and primer selection for PCR-based targeted markerless allelic replacement mutagenesis of the *hla* gene encoding α-toxin in methicillin-resistant SA strain USA300 TCH1516.

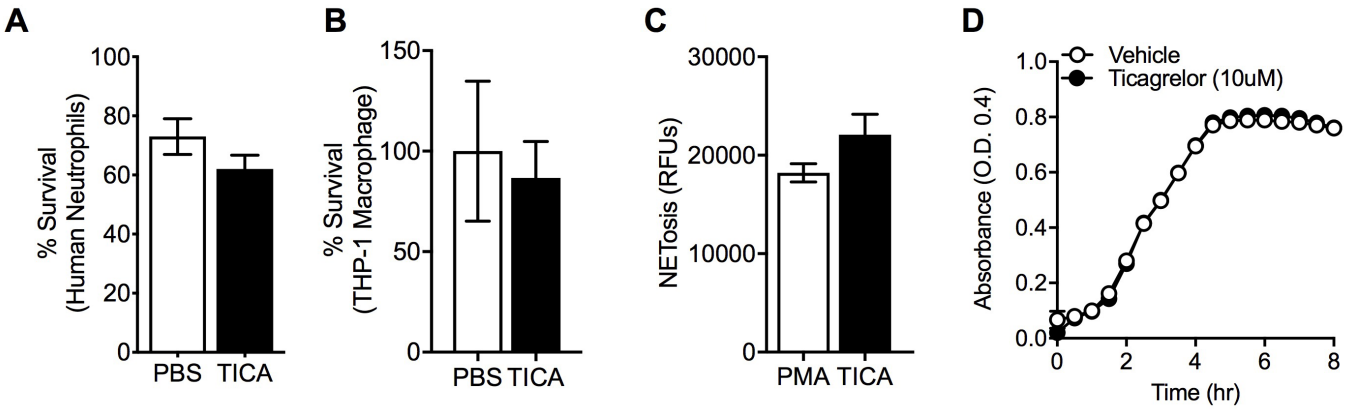


Fig. S4. Exclusion of ticagrelor off-target effects on SA immune cell interactions and growth. (**A**) Quantification of MRSA colony-forming units (CFUs) in a purified human neutrophil killing assay. Human neutrophils and (**B**) THP-1-derived macrophages treated with vehicle control (PBS 1x) or 10 μM ticagrelor (TICA) for 30 minutes prior to 1 h exposure to MOI: 1 MRSA. (**C**) Isolated human neutrophils pre-treated with vehicle or 10 μM TICA and subsequently exposed to PMA for quantification of neutrophil extracellular trap (NET) production. (**D**) Growth curve analysis of pre-treated SA with 10μM TICA. Absorbance measured at an optical density (O.D.) of 0.4 measured over the course of 8 hours. Where applicable, data represented as mean ± SEM and are representative of at least three independent experiments. Statistical significance determined by unpaired two-tailed Student's t test. *$P < 0.05$

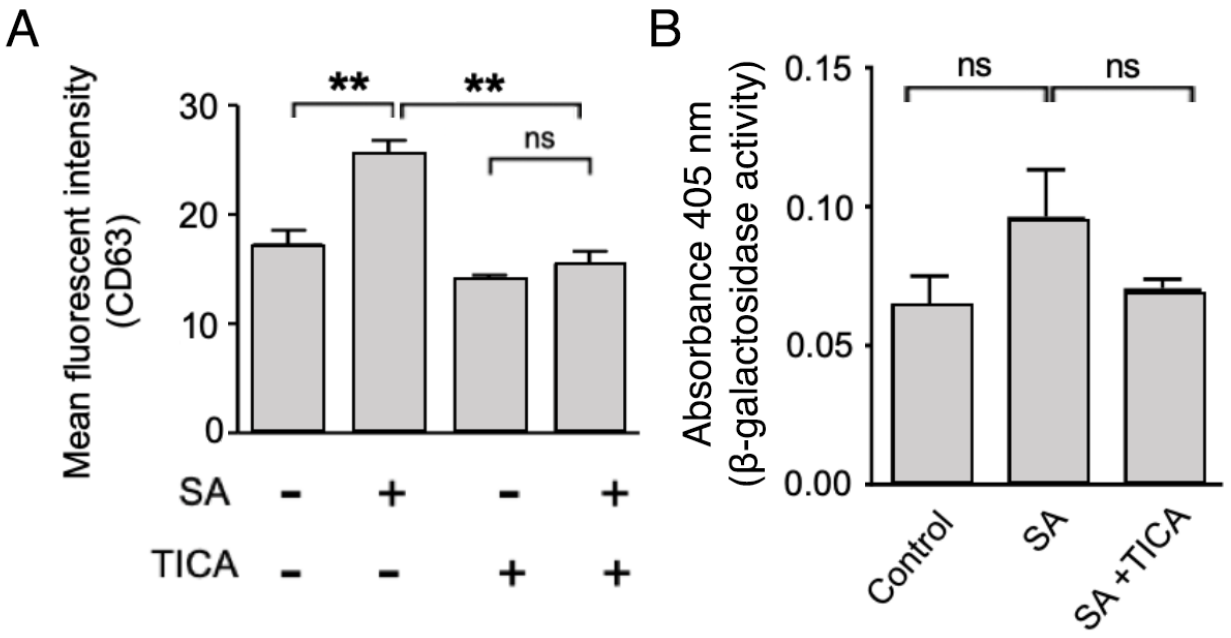


Fig. S5. Additional effects of SA or TICA on human platelet phenotypes in vitro. (**A**) Human platelet CD63 expression was measured by flow cytometry with or without TICA treatment and MRSA challenge (MOI = 0.1) after 90 min. (**B**). β-Galactosidase activity released from human platelets were measured using the colorimetric method of the Mammalian β-Galactosidase Assay Kit (Thermo-Fisher). All data are represented as mean ± SEM and are representative of at least 3 independent experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's multiple comparisons test (A,C,G), unpaired two-tailed Student's t-test (H), and two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons posttest (I).

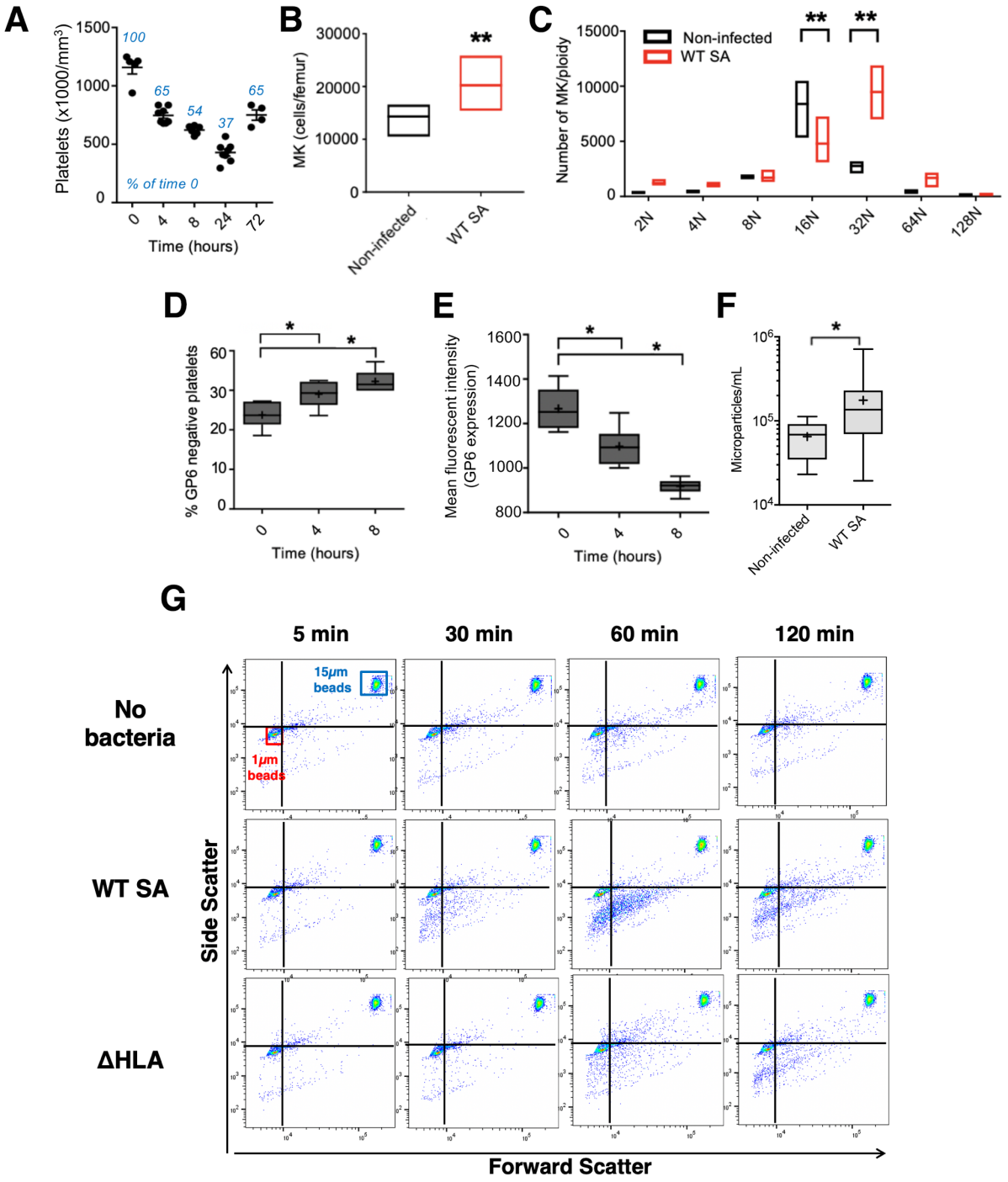
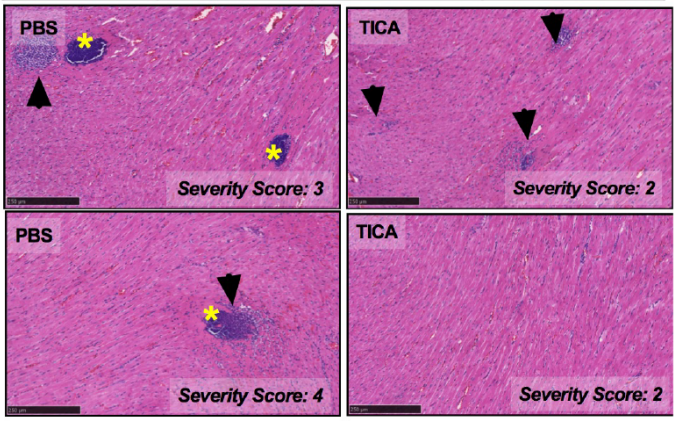


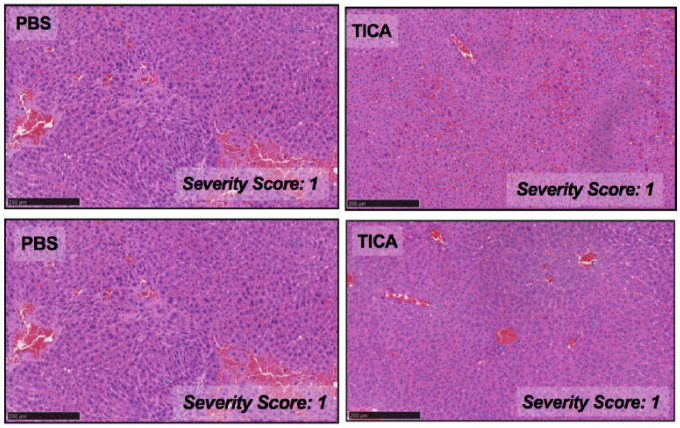
Fig. S6. Effects of SA challenge on platelets and thrombopoiesis in vivo. (**A**) Platelet counts after inducing systemic SA infection (5 x 10^7 CFU SA i.v.). (**B and C**) Bone marrow cells were harvested after 72 h of SA systemic infection and analyzed for megakaryocyte count (**B**) and ploidy distribution (**C**). (**D and E**) Circulating platelets were analyzed for % GP6 negative platelets (**D**) and surface GP6 expression (**E**) by flow cytometry after inducing systemic SA infection (5 x 10^7 CFU SA i.v.). (**F**) Platelet microparticulation was measured in vivo. Mice were injected 1 x 10^8 SA i.v. and after 4 h, blood was

collected and analyzed for platelet microparticles by flow cytometry. (**G**) Human platelets were infected with SA in vitro. Platelet microparticles in the samples were analyzed at each time point by flow cytometry.

Mouse	Organ	Colony Count	Severity Score
PBS 1	Heart	35	3
PBS 2	Heart	32	4
TICA 1	Heart	6	2
TICA 2	Heart	3	2



Mouse	Organ	Colony Count	Severity Score
PBS 1	Liver	N/A	1
PBS 2	Liver	N/A	1
TICA 1	Liver	N/A	1
TICA 2	Liver	N/A	1



Mouse	Organ	Colony Count	Severity Score
PBS 1	Spleen	N/A	2
PBS 2	Spleen	N/A	3
TICA 1	Spleen	N/A	2
TICA 2	Spleen	N/A	2

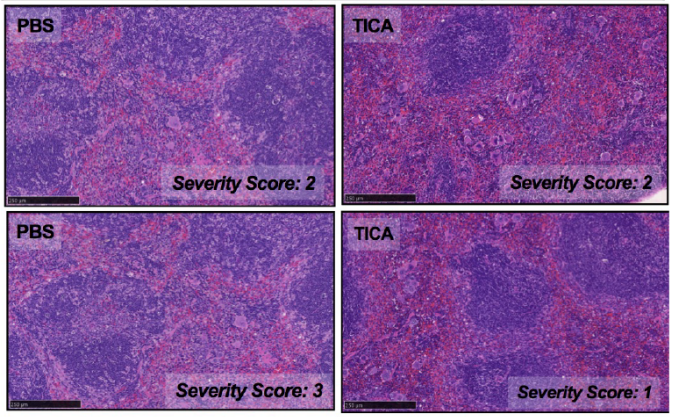


Fig. S7. Organ pathology in murine SA infection with or without ticagrelor treatment. Hematoxylin and eosin stain (H&E) of representative histological heart, liver, and spleen sections from mice pre-treated with vehicle or 4 mg/kg ticagrelor 12 h prior to SA infection and q 12 h thereafter for 72 h; (n = 2). Yellow stars denote dense bacterial colonies. In the ticagrelor-treated mice, the bacterial colonies were smaller, less frequent, and often surrounded by an inflammatory infiltrate (black arrow) comprising neutrophils with fewer macrophages. Images are representative of at least two independent experiments. Scale bars = 250 μM.

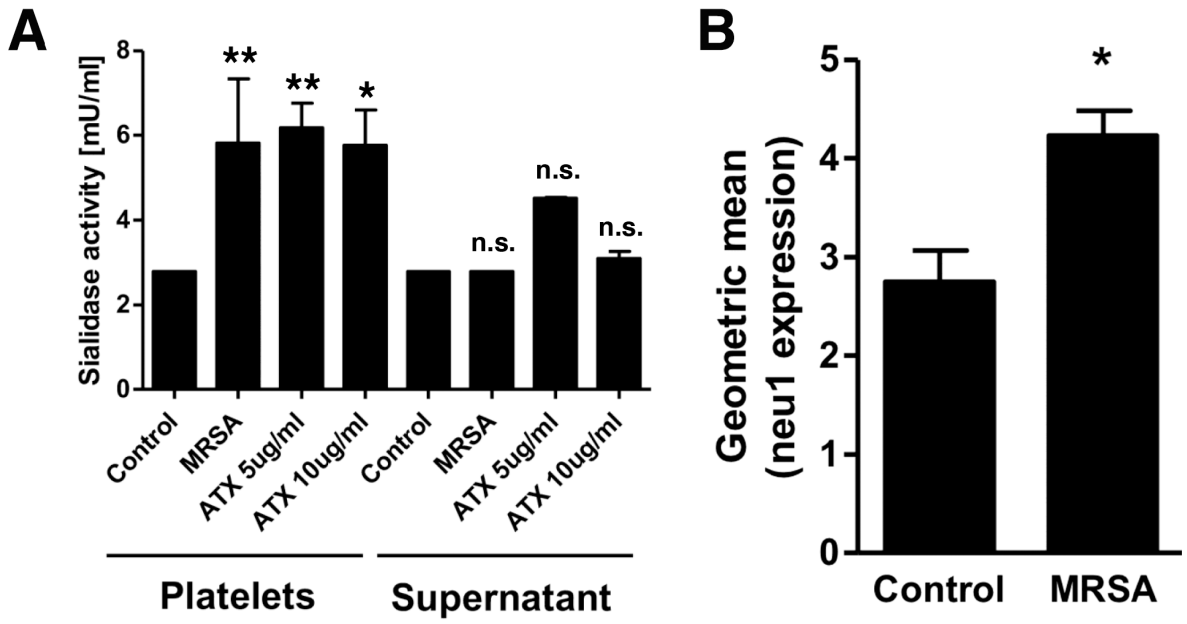


Fig. S8. Neu1 is predominantly detected on the platelet cell surface and is induced by SA exposure. (**A**) Human platelet-rich plasma was incubated with 2 x 10^7 CFU SA or with 5 or 10 μg/ml recombinant α-toxin (ATX). Following 30 min incubation at 37ºC, platelets and plasma were separated by centrifugation. Both fractions were assayed for sialidase activity using the Amplyx Red Assay (Fisher Scientific) per manufacturer's instructions, using DANA as a background control. A standard curve was established using *Arthrobacter ureafaciens* sialidase (AUS), with a starting concentration of 500 mU/mL serially diluted down to 0.98 mU/mL. (**B**) Human platelets were incubated with WT SA at MOI = 1 for 30 min rotating at 37ºC, then incubated with anti-Neu1 antibody, followed by addition of AlexaFluor 488 goat anti-rabbit IgG. Samples were washed and the surface expression of Neu1 analyzed by flow cytometry. Data are shown as mean ± SEM and are representative of at least three independent experiments. Statistical significance determined by unpaired two-tailed Student's t test. *$P < 0.05$

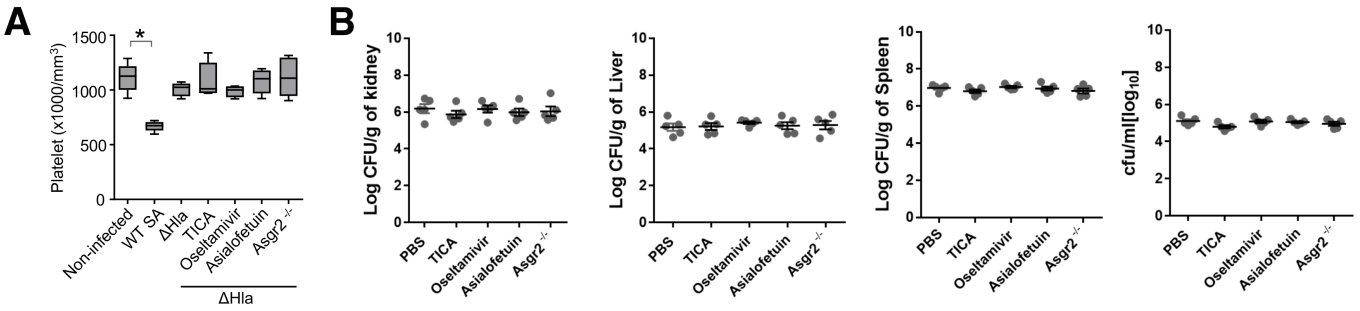


Fig. S9. SA α-toxin deletion mutant phenocopies protective effects of ticagrelor, oseltamivir, and AMR loss or inhibition. (A) WT C57/Bl6 mice or *Asgr2*$^{-/-}$ mice were either challenged with 8 x 10^8 WT SA or its isogenic ΔHla mutant. C57/BL6 mice infected with ΔHla were subsequently treated with TICA, oseltamivir, asialofetuin, or PBS control. Blood was harvested 4 h post infection and **(A)** platelets or **(B)** bacterial colony forming units (CFU) enumerated. Statistical significance determined by unpaired Two-tailed Student's t-test. For floating bar graphs, - denotes the median, whiskers represent min. to max, and floating box represents the 25th to 75th percentile. Unless otherwise stated, *P < 0.05. PBS, phosphate buffered saline; ns, not significant; WT, wild-type; TICA, ticagrelor.

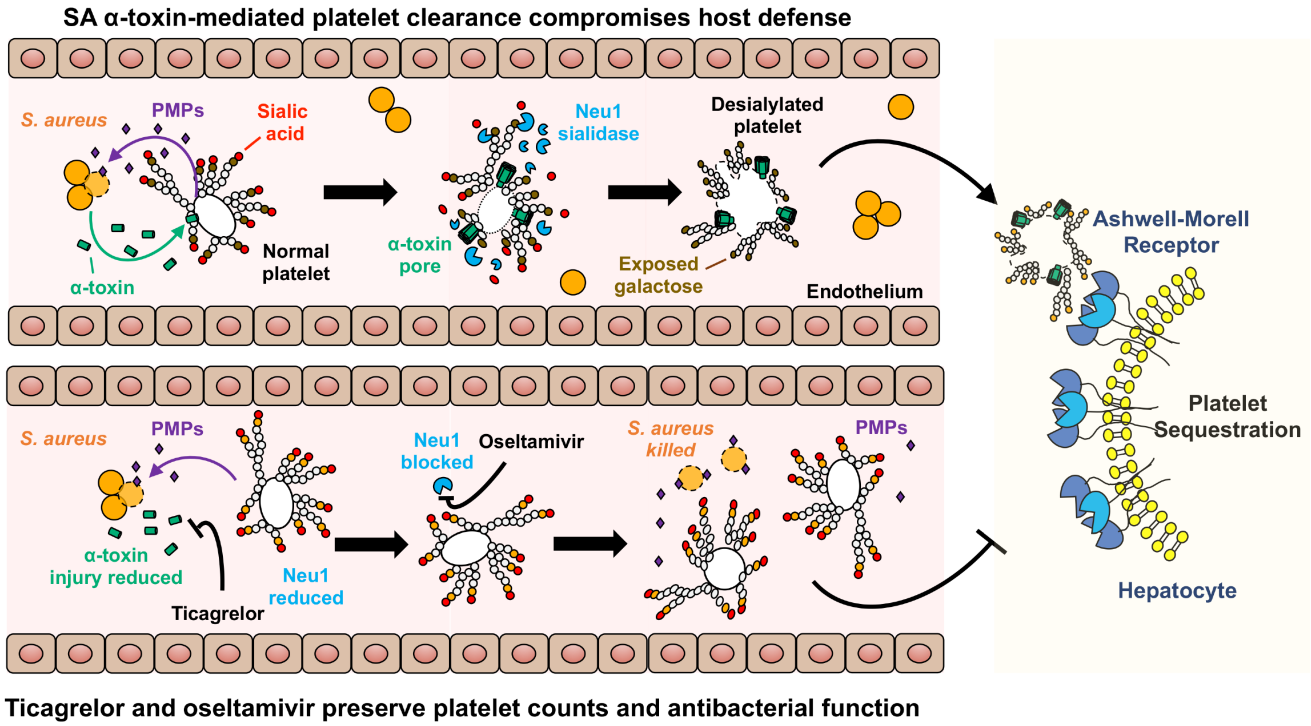


Fig. S10. Schematic illustration of proposed "toxin-platelet-AMR" pathway exploited by SA in the pathogenesis of bloodstream infection. Our experiments suggest the possibility of therapeutic drug repurposing of P2Y12 (for example ticagrelor) and sialidase inhibitor (for example oseltamivir) drugs to maintain platelet homeostasis and enhance innate immune clearance of the pathogen.

The following files can be found in the online version of the supplement:

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