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PRECLINICAL RESEARCH

Protective Effect of Ticagrelor Against Infective Endocarditis Induced by Virulent *Staphylococcus aureus* in Mice

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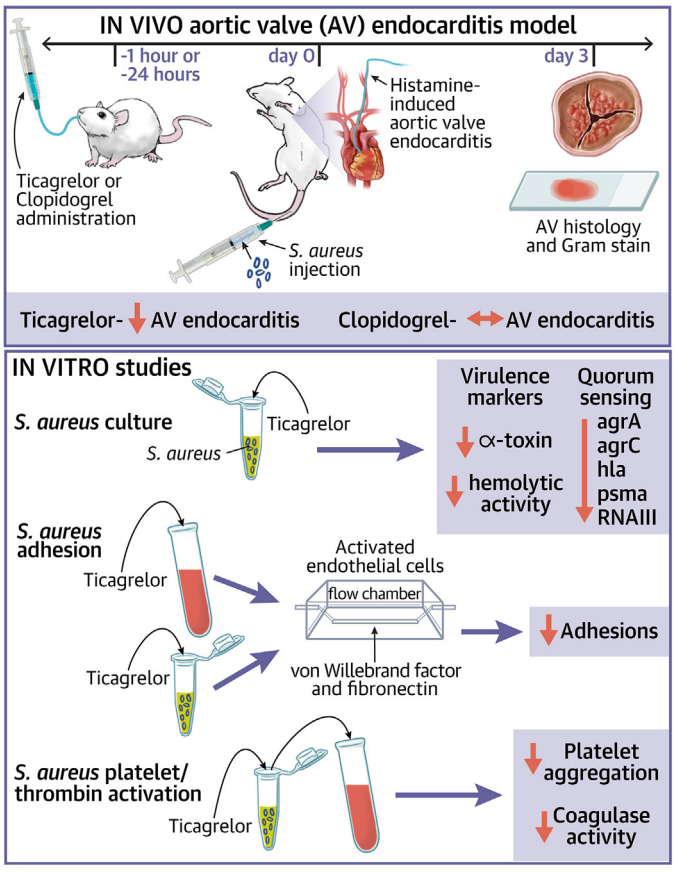
VISUAL ABSTRACT



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HIGHLIGHTS

- **In addition to its potent antiplatelet activity, ticagrelor possesses antibacterial properties against Gram-positive bacteria. We wondered whether the typical clinical dosage of ticagrelor could prevent the development of IE caused by highly virulent *S aureus*.**
- **A single antiplatelet dose of ticagrelor prevented *S aureus*-infected vegetation formation in a mouse model of inflammation-induced endocarditis. The dosage achieved in patients under ticagrelor therapy altered bacterial toxin production and adherence on activated ECs, thereby mitigating bacterial virulence.**
- **Besides the previously described bactericidal activity at high doses, ticagrelor at typical clinical doses possesses antivirulence activity against *S aureus*, which might be beneficial for the prevention of IE. Ticagrelor antiplatelet activity may further interfere with the interplay between platelets and bacteria.**

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ABBREVIATIONS AND ACRONYMS

ACS = acute coronary syndrome

Agr = accessory gene regulator

CFU = colony-forming units

DAPT = dual antiplatelet therapy

ΔHla = α-toxin-deficient *Staphylococcus aureus* JE2 strain

Δ*vwb* = von Willebrand binding protein-deficient *Staphylococcus aureus* JE2 strain

Δ*coa* = coagulase-deficient *Staphylococcus aureus* JE2 strain Δ*vwb*Δ*coa*

EC = endothelial cells

Hla = α-toxin

HUVEC = human umbilical vein endothelial cells

IE = infective endocarditis

mRNA = messenger RNA

MRSA = methicillin-resistant *Staphylococcus aureus*

PRP = platelet-rich plasma

PVL = Panton-Valentine leucocidin

TSB = Tryptic Soy Broth

VWF = von Willebrand factor

SUMMARY

In addition to its potent antiplatelet activity, ticagrelor possesses antibacterial properties against gram-positive bacteria. We wondered whether the typical clinical dosage of ticagrelor could prevent the development of infective endocarditis caused by highly virulent *Staphylococcus aureus*. Ticagrelor prevented vegetation formation in a mouse model of inflammation-induced endocarditis. The dosage achieved in patients under ticagrelor therapy altered bacterial toxin production and adherence on activated endothelial cells, thereby mitigating bacterial virulence. Besides the previously described bactericidal activity at high doses, ticagrelor at typical clinical doses possesses antivirulence activity against *S aureus*. Ticagrelor antiplatelet activity further interferes with the interplay between platelets and bacteria. (J Am Coll Cardiol Basic Trans Science 2023;■:■–■)

Ticagrelor is an orally active cyclopentyl-triazolopyrimidine antiplatelet drug acting by reversibly inhibiting the platelet P2Y12 receptor.[1] It is indicated to prevent cardiovascular events in patients with coronary artery disease and peripheral vascular disease. Owing to the high prevalence of these diseases and accumulating evidence for the high clinical performance of ticagrelor over other P2Y12 inhibitors (ie, clopidogrel), it is currently one of the most administered drugs worldwide.

In addition to its potent antithrombotic effect, we discovered, in 2019, that ticagrelor has bactericidal activity against gram-positive bacteria resistant to conventional antibiotics, including methicillin-resistant *S aureus* (MRSA), glycopeptide-intermediate *S aureus*, and vancomycin-resistant *Enterococcus faecalis*, pathogens that pose the greatest threat to human health according to the World Health Organization.[2] Our findings have been put forward as the first possible explanation of data from a subanalysis of the Platelet Inhibition and Patient Outcomes (Comparison of Ticagrelor [AZD6140] and Clopidogrel in Patients With Acute Coronary Syndrome) clinical trial, showing that ticagrelor therapy was associated with a lower risk of death related to infection as compared with clopidogrel.[3] An antibacterial effect could also explain the improvement of lung function by ticagrelor in pneumonia patients of the small XANTHIPPE (Targeting Platelet-Leukocyte Aggregates in Pneumonia With Ticagrelor) study.[4] More recently, a study by Ulloa et al[5] described the successful use of ticagrelor as an adjuvant therapy to antibiotics in a case report of a male patient with *S aureus* endovascular infection and multiple hematogenous infectious foci. Furthermore, 2 studies reported that ticagrelor therapy in patients with coronary artery disease who experienced an ST-segment elevation acute myocardial infarction was associated with a reduced risk of infection as compared with clopidogrel or prasugrel.[6,7]

Noteworthy, in our in vitro antibacterial assays, ticagrelor minimal bactericidal concentrations against the gram-positive organisms tested were approximately 20 mg/L, which is well above the concentrations reached in conventionally dosed patients treated for cardiovascular diseases (ticagrelor maximum concentration = 1.2 mg/mL after one 180-mg loading dose and 0.75 mg/mL at 90 mg twice

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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daily steady state). Nevertheless, treating mice with conventional oral antiplatelet dosage of ticagrelor could inhibit *S aureus* growth on a preinfected subcutaneous implant.[2] Likewise, in a recent study in mice, a similar ticagrelor dosage could protect mice against MRSA bacteremia, leading to a decreased bacterial load in the blood, kidney, liver, and spleen.[8] Therefore, although clinical observations and data in mice support the antibacterial activity of standard ticagrelor dosage, the underlying mechanisms, including a role for platelets, remain unclear.

Infective endocarditis (IE) is a life-threatening infectious disease affecting native heart valves or prosthetic valves[9] that is associated with a very high 1-year mortality rate of approximately 30% to 40%.[10] IE can affect ≤3% of patients with a prosthetic valve, and its prevalence is expected to increase owing to steadily increasing numbers of heart valve implantations (estimated to reach 850,000 per year by 2050). The main agents responsible for IE are gram-positive bacteria, with *S aureus* being the most frequent and virulent. IE is characterized by the formation of a vegetation on the heart valve surface that mainly consists of bacteria, platelets, and fibrin. Current antibiotic-based treatments against IE lack efficacy and the situation is worsening owing to the emergence of multidrug-resistant bacteria. Therefore, there is a substantial need for new strategies that could prevent or treat IE.

In the present study, we tested the ability of ticagrelor or clopidogrel to prevent *S aureus* IE by using a previously described mouse model of the disease. We then investigated the mechanisms of direct antibacterial effects of ticagrelor conventional dosage underpinning mitigated *S aureus* virulence and adherence at disease initiation.

METHODS

ETHICS STATEMENTS. Blood samples were obtained by venipuncture from healthy volunteers according to the European Union regulation on the collection and use of samples of human body material for research purposes. The study has been approved by the Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège (2021-109). All healthy volunteers have signed an informed consent document before blood donation. The experiments with animals were carried out in strict accordance with the guidelines for the care and use of animals set out by the European Union (Directive 2010/63/EU). The mouse model of IE has been reviewed and approved by the KU Leuven Animal Ethics Committee (license number 189/2017).

BACTERIA STRAINS AND GROWTH CONDITIONS. All experiments performed with live *S aureus* were performed under biosafety level 2 conditions with appropriate safety precautions for staff. Bacterial strains included a previously characterized IE clinical isolate,[11] JE2 (USA300 MRSA), α-toxin-deficient JE2 bacteria JE2 strain (ΔHla) (Nebraska *S aureus* Transposon Mutant Library), von Willebrand binding protein-deficient JE2 (Δ*vwb*), coagulase-deficient JE2 (Δ*coa*), and Δ*vwb*Δ*coa* JE2 strains.[11] Bacteria were grown in Tryptic Soy Broth (TSB, Sigma) under agitation (200 rpm) at 37°C. Before all experiments, a single colony from Tryptic Soy Agar plates was used to inoculate 4 mL of TSB in 15-mL polystyrene tubes and bacteria were grown overnight. Liquid cultures were then diluted 100-fold in 20 mL fresh TSB and aliquoted in new tubes. Ticagrelor or vehicle (1% dimethyl sulfoxide) was added to bacteria suspensions and bacterial growth was recorded at regular time intervals (OD_{600}). Unless specified, bacteria were used at exponential growth phase, pelleted, and washed to eliminate free ticagrelor. Supernatants from stationary phase bacteria were collected by centrifugation and filtered through a 0.22-μM cellulose acetate filter (VWR) and kept at -20°C until further use.

MOUSE MODEL OF IE. One hour before infection, mice were gavaged with 3 mg/kg ticagrelor (Cayman Chemical) or 4% dimethyl sulfoxide vehicle control. In a separate set of experiments, mice received 30 mg/kg clopidogrel (Eurogenerics) or vehicle (0.003% v/v HCl in water) 24 hours before infection. Mice were injected with *S aureus* clinical isolate (2×10^6 CFU/mouse) via the tail vein just before administering a 200-mmol/L histamine infusion (infusion rate of 10 μL/min for 5 minutes; Sigma-Aldrich) locally at the aortic valve through a 32G polyurethane catheter introduced in the carotid artery. Afterward, the catheter was removed, and the surgical site was closed with surgical sutures. The mice were monitored subsequently up to day 3 with a scoring system to assess animal well-being, including weight loss, activity levels, and breathing quality. At experimental or humane endpoints, before euthanasia, blood was withdrawn via retro-orbital puncture and plated on mannitol salt agar plates to define bacteremia levels by counting colony-forming units (CFU), and hearts were perfused with 0.9% sodium chloride followed by 4 % paraformaldehyde. Paraffin sections of the hearts were prepared and stained with Brown-Hopps Gram stain for analysis of endocarditis and presence of bacteria by an investigator blinded to the sample identities. Bacteria content was further

evaluated by immunofluorescence staining using a primary anti-*Staphylococcus aureus* antibody from Abcam (ab20920), and platelet and leucocyte content by immunohistochemistry using anti-CD61 (Cell Marque, CMC 42490030) and anti-CD45 (Abcam, ab10558) antibodies, respectively. Fibrin content was visualized by Martius Scarlet blue staining. The signal positive area was quantified using Image J software (NIH). The method of determination of ticagrelor concentration in mouse plasma is described in the Supplemental Appendix.

PREPARATION OF RED BLOOD CELL SUSPENSIONS AND HEMOLYTIC ASSAYS. Freshly drawn citrate anticoagulated blood from healthy volunteers was centrifugated for 15 minutes at 1,000×*g*. After discarding the plasma upper layer, red blood cells were washed once and resuspended in phosphate-buffered saline. Bacterial supernatants from were mixed with freshly prepared human red blood cell suspensions to a 1:5 final ratio and incubated for 30 minutes under gentle agitation (800 rpm). Samples were then centrifuged at 8,000×*g* for 5 minutes. Hemolytic activity was determined in supernatants by measuring the absorbance at 570 nanometers.

WESTERN BLOTTING EXPERIMENTS. Bacterial supernatants were mixed with Laemmli buffer, proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and electrotransferred on polyvinylidene difluoride membranes. The primary antibodies against α-toxin and Panton-Valentine leucocidin (PVL) were purchased from Abcam. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare and Cell Signaling, respectively. Quantification of α-toxin and PVL expression levels was performed by densitometry using Image J (NIH).

ANALYSIS OF STAPHYLOXANTHIN PRODUCTION. Pellets from stationary phase bacteria were resuspended in 400 mL methanol and incubated for 30 minutes at 37°C under gentle agitation. Samples were centrifuged for 5 minutes at 8,000×*g* to remove cell debris. Pigment intensity in supernatants was analyzed by measuring the absorbance at 470 nanometers.

RNA EXTRACTION AND QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION. Bacteria were grown in the presence of ticagrelor or vehicle under agitation for 6 hours (midexponential growth phase). Bacterial RNA was extracted using the RNAprotect Bacteria Reagent and RNeasy mini kit (Qiagen) following the manufacturer's instructions. After DNase treatment, purified RNA was reverse transcribed (RevertAid H minus first strand, Thermo Fisher Scientific) and subjected to real-time PCR using SYBR premix (Takara). Primers are listed in Supplemental Table 1. Gene expression levels were calculated with the $\Delta\Delta C_t$ methods and normalized to *gyrB* (DNA gyrase subunit B) expression levels.

BACTERIA ADHESION ASSAYS. Plates were coated with human plasma fibrinogen (50 mg/mL) (Merck) for 24 hours before blocking with 2% bovine serum albumin for 1 hour. We then added 10^6 CFU/mL 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester-labelled bacteria and incubated for 30 minutes at 37°C. Adherent bacteria were fixed with 4% paraformaldehyde and fluorescence intensity was quantified.

PLATELET AGGREGATION ASSAYS. Platelet aggregation was recorded by light transmission aggregometry (Chrono-Log) after adding 10-fold concentrated bacterial supernatants or live bacteria (1:10 bacteria:platelet ratio) to human citrated platelet-rich plasma (PRP) at 37°C under stirring (1,200 rpm). Additionally, PRP collected from ticagrelor and clopidogrel treated mice was stimulated with 10 μM adenosine diphosphate.

CLUMPING ASSAY. *S aureus* clumping was analyzed over time by light transmission aggregometry by adding citrate-anticoagulated human plasma to bacterial suspensions (1% v/v) at 37°C under stirring conditions (1,200 rpm).

PLATELET-KILLING ASSAY. Bacteria grown in the presence of ticagrelor or vehicle were pelleted by centrifugation and washed in Roswell Park Memorial Institute medium. Washed platelets were resuspended in the same medium at a density of 250,000 platelets/mL and incubated with bacteria at a ratio of 10 to 1 for 90 minutes before plating and CFU counting.

FLOW CHAMBER ASSAYS. Glass coverslips (24 × 60 mm) were coated with 1% rat tail collagen type I (Sigma-Aldrich) for 1 hour at 37°C. Human umbilical vein endothelial cells (HUVECs) from pooled donors (Lonza) were cultured in complete medium (EBM-2, Lonza). We seeded 300,000 cells/well on the coated coverslip to obtain a cell coverage of approximately 70% to 80% after 4 days of growth. HUVECs were activated with 25 ng/mL recombinant human tumor necrosis factor-α (R&D systems) for 16 hours. The coverslips were mounted in a dedicated flow chamber system as described.[12] Activated HUVECs were perfused at a shear rate of 1,000 s^{-1} for 10 minutes with 1 mL anticoagulated blood containing 10^8 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester-

labelled bacteria and an Alexa Fluor 647 anti-human CD42b antibody for platelet detection (Biolegend). Images were captured after 10 minutes of perfusion using an inverted fluorescence EVOS microscope (Bothel) at a magnification of ×60. Bacteria and platelet adhesion were quantified by measuring fluorescence intensity on 10 images with the QuPath software. For the analysis of bacteria adhesion to von Willebrand factor (VWF) under flow, glass coverslips (24 × 60 mm) were coated with 50 μg/mL VWF (Haemate P, CSL Behring) for 4 hours at room temperature before perfusing a suspension of 10^8 labeled bacteria/mL at a shear rate of 1,000 s^{-1} for 10 minutes.

THROMBIN ACTIVITY ASSAY. Bacterial supernatants were concentrated ten times using a Pierce Protein Concentrator PES, 10K MWCO (Thermo Fisher Scientific). Thrombin activity was measured at 37°C for 5 hours after addition of 0.2 mmol/L Thrombin Chromogenic Substrate (Innovative Research, IAFTHRCGSLY25MG) to a mixture of bacterial supernatant and human pooled plasma (cryocheck, PrecisionBiologic).

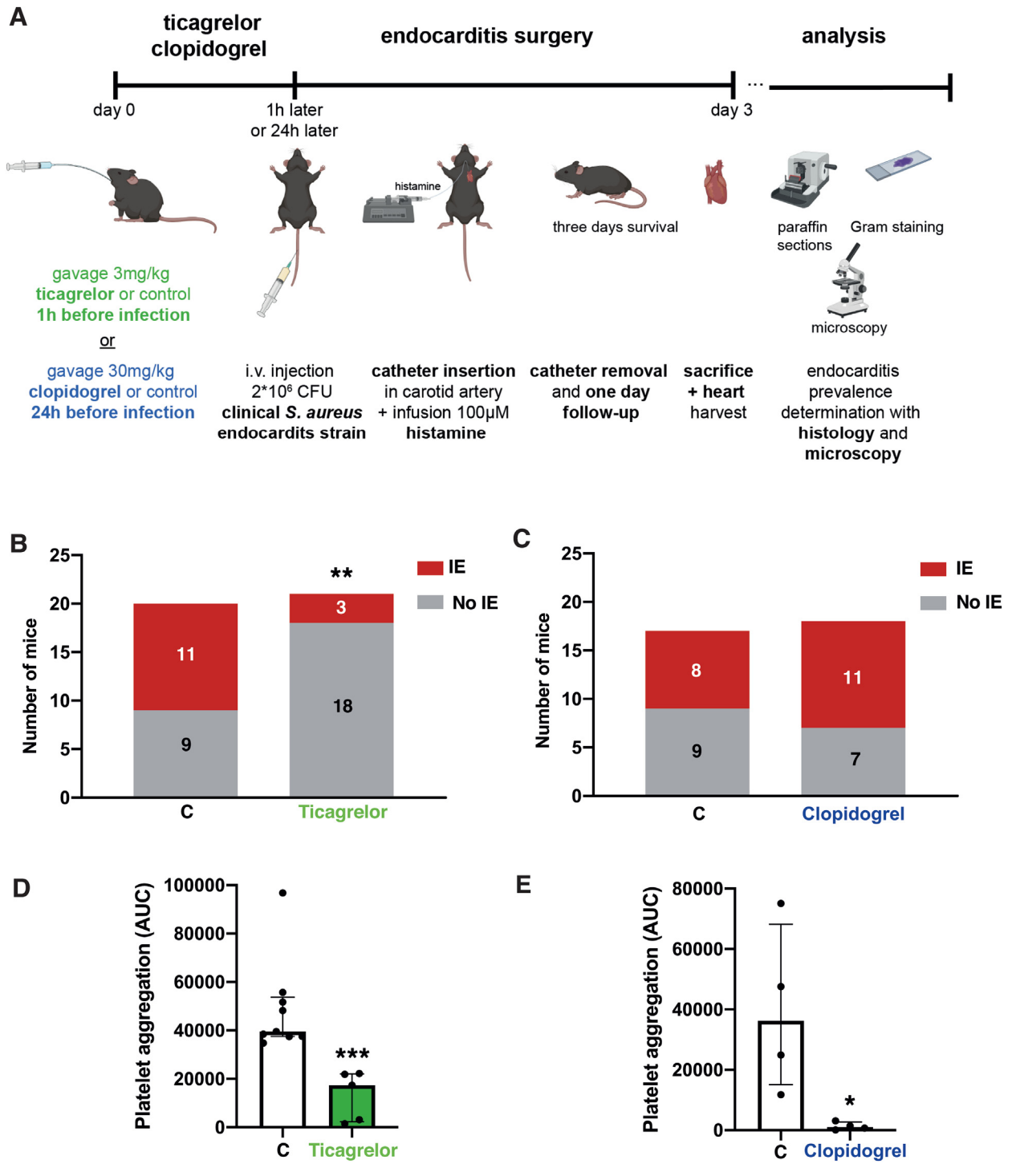
STATISTICAL ANALYSIS. Data are reported as mean ± SD or median with 25th and 75th percentiles (Q1-Q3). Normality of the data was assessed by the Shapiro-Wilk test. Fisher's exact test or the chi-square test was used to analyze the effect of treatments on in vivo IE development. The Mann-Whitney *U* test was used to investigate ex vivo platelet aggregation between control and treated groups. To determine whether differences existed among more than 2 treatments, a 1-way analysis of variance with post hoc Dunnett's test or Kruskal-Wallis with post hoc Dunn's test for multiple pairwise comparisons were used when appropriate. When paired data from the same sample were compared, a repeated measures analysis of variance was used. Comparisons between the 2 groups were analyzed using Student's *t*-test for independent samples or paired *t*-tests for dependent samples. Relative messenger RNA (mRNA) expression was analyzed by a 1-sample *t*-test. All tests are 2-sided and a *P* value of <0.05 was considered significant. Kaplan-Meier survival plots of mice receiving treatments and vehicle were compared by the log-rank test. Statistical analysis was performed using GraphPad Prism 8.2 Software.

RESULTS

TICAGRELOR BUT NOT CLOPIDOGREL PREVENTS *S AUREUS* IE DEVELOPMENT. We initiated *S aureus* IE upon histamine-induced inflammation as previously described[11] and subjected mice to pharmacologic P2Y12 inhibition (**Figure 1A**). Under these experimental conditions,[11] approximately one-half (55%) of control mice (receiving ticagrelor vehicle) developed IE. In contrast, IE was present in only 3 of 21 (14.3%) mice that received ticagrelor (**Figure 1B**) ($P = 0.009$, ticagrelor vs vehicle). In these 3 mice, thrombus size, bacteria, platelet, leucocyte, and fibrin content did not differ from those in control mice with IE (Supplemental Figure 1). We assessed whether IE prevention with ticagrelor could be due to its platelet inhibitory activity by treating mice with another P2Y12 inhibitor, clopidogrel. Because clopidogrel is a prodrug that needs to be metabolized to be active, mice were gavaged with clopidogrel or vehicle 24 hours before bacterial infection and surgery (**Figure 1A**). Mice that received clopidogrel were not protected from IE development, with 61.1% of mice having IE (n = 18) (**Figure 1C**). Thrombus size, bacteria, and fibrin content did not differ between the clopidogrel and control mice with IE (Supplemental Figure 2). Neither CFU count in blood nor mouse survival significantly differed between control and ticagrelor- or clopidogrel-treated mice (Supplemental Figures 3 and 4). Platelet inhibition was verified in independent ex vivo adenosine diphosphate-induced platelet aggregation assays performed 1 and 24 hours after gavage with ticagrelor and clopidogrel, respectively (**Figures 1D and 1E**). In agreement with ticagrelor pharmacokinetics in mice and its reversible mode of action,[13] neither ticagrelor molecule nor its antiplatelet activity was detected beyond 2 hours after gavage (Supplemental Figure 3). Altogether, these data indicate that platelet inhibition alone is unlikely to explain IE prevention by ticagrelor.

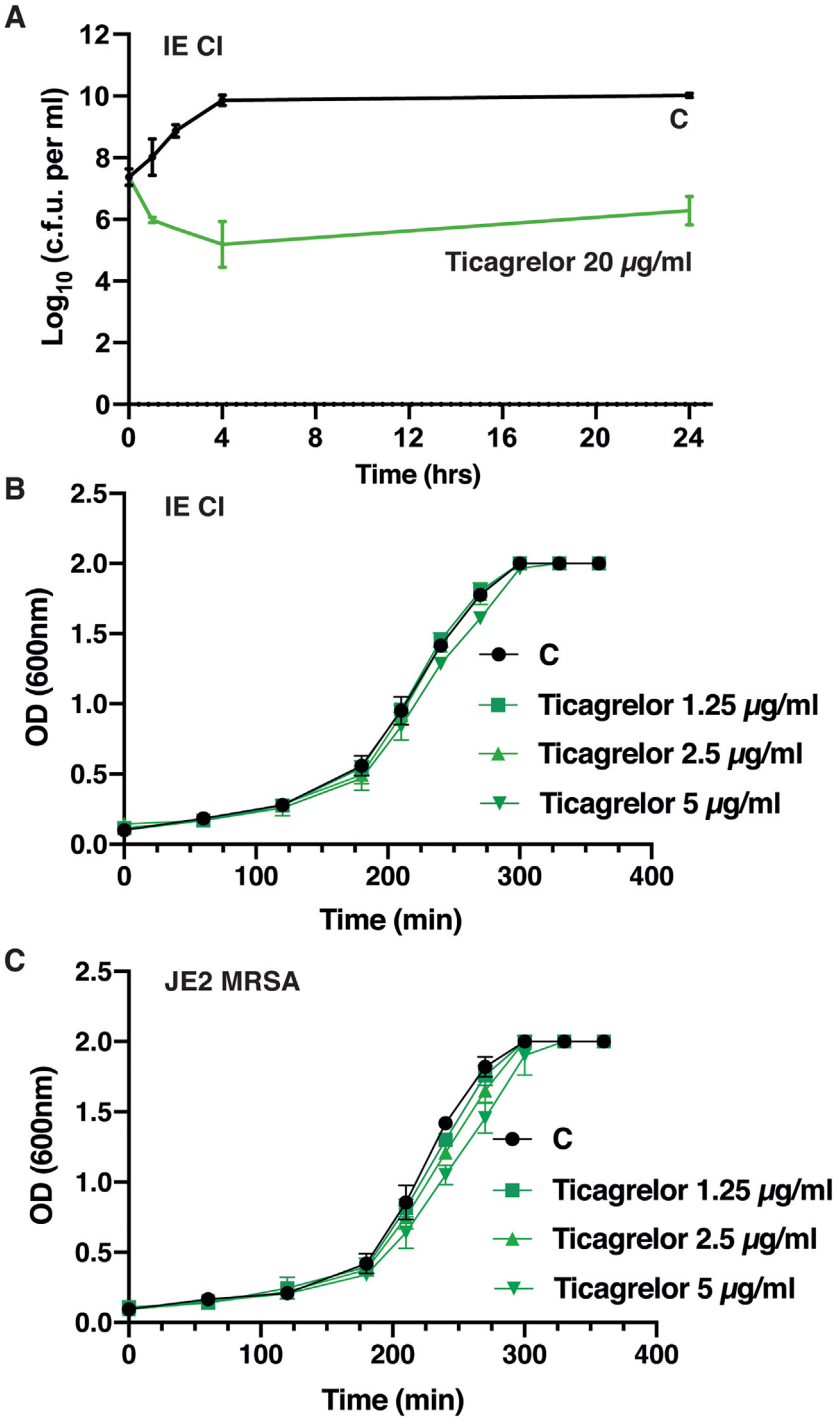
CONVENTIONAL CLINICAL DOSAGE OF TICAGRELOR DOES NOT INHIBIT *S AUREUS* BACTERIAL GROWTH. We then investigated the direct effect of ticagrelor on bacteria. We first evaluated the susceptibility of the used IE *S aureus* clinical strain to ticagrelor by determination of minimal inhibitory and bactericidal concentrations according to the European Committee on Antimicrobial Susceptibility standards. The minimal inhibitory concentration and minimal bactericidal concentration of ticagrelor against this strain were both 20 mg/mL, which does not differ from values reported in our initial study using laboratory strains of methicillin-sensitive *S aureus* and MRSA.[2] A time-kill assay performed at the minimal bactericidal concentration of ticagrelor also revealed similar kinetics of action, as in our previous report (**Figure 2A**). We further showed that ticagrelor plasma concentrations consistent with those obtained in conventionally dosed patients (1.25 mg/mL) and ≤5 mg/mL did not affect bacterial growth of the used IE clinical

FIGURE 1 Ticagrelor But Not Clopidogrel Prevents *S aureus* IE Development



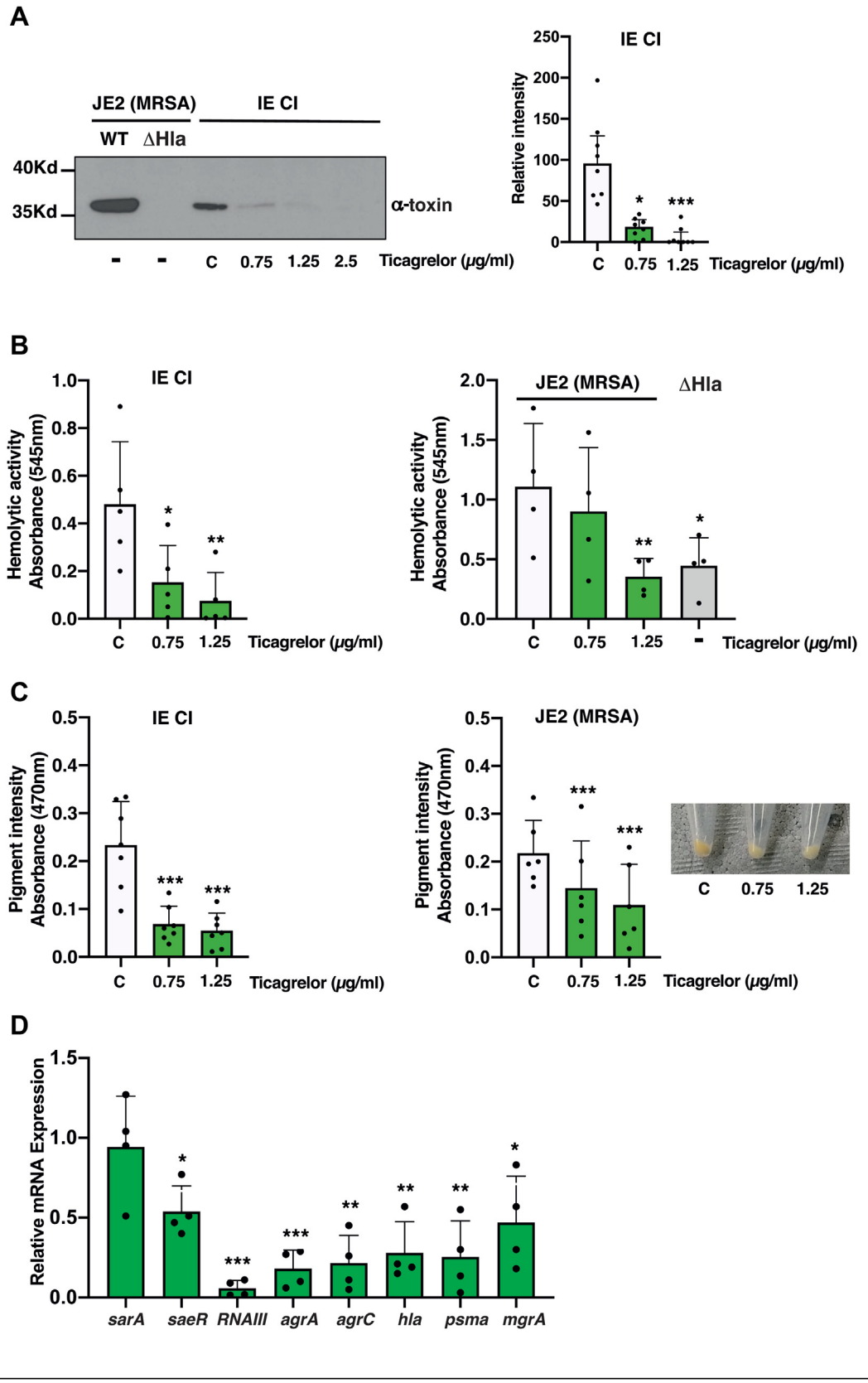
(A) Experimental setup. Mice received a single oral dose of ticagrelor or clopidogrel or corresponding vehicles before surgical catheter insertion, histamine infusion on the surface of aortic valve and intravenous injection of a *S aureus* IE CI. This figure was created with Biorender. **(B)** Number of ticagrelor-treated and control mice that developed IE (**$P < 0.01$; Fisher's exact test). **(C)** Number of clopidogrel-treated and control mice that developed IE ($P > 0.05$; chi-square test). (D) Ex vivo ADP (10 mmol/L)-induced platelet aggregation analyzed in PRP 1 hour after oral administration of ticagrelor (3 mg/kg, n = 5) or vehicle (n = 9). Data represent median and 25th to 75th percentiles (***$P = 0.001$, Mann-Whitney *U* test). **(E)** Ex vivo ADP (10 mmol/L)-induced platelet aggregation analyzed in PRP 24 hours after oral administration of clopidogrel (30 mg/kg, n = 4) or vehicle (n = 4). Data represent median and interquartile range (*$P = 0.03$, Mann-Whitney *U* test). ADP = adenosine diphosphate; AUC = area under the curve; CI = clinical isolate; CFU = colony-forming units; IE = infective endocarditis; PRP = platelet-rich-plasma; *S aureus* = *Staphylococcus aureus*.

FIGURE 2 **Ticagrelor Antibacterial Activity**



(A) Time-kill curve of *S aureus* IE CI with ticagrelor (20 μg/mL) or vehicle (**C**: 1% DMSO) (n = 3). **(B)** Growth of IE CI was followed over time in TSB medium containing the indicated concentrations of ticagrelor or vehicle (**C**: 1% DMSO). **(C)** Growth of USA300 JE2 MRSA was followed over time in TSB medium containing the indicated concentrations of ticagrelor or vehicle (**C**: 1% DMSO). Data are from 3 independent experiments. DMSO = dimethyl sulfoxide; MRSA = methicillin-resistant *Staphylococcus aureus*; OD = optical density; TSB = Tryptic Soy Broth; other abbreviations as in Figure 1.

FIGURE 3 **Conventional Concentrations of Ticagrelor Inhibit Toxin Production by *S aureus***



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strain or of the USA300 JE2 strain of MRSA (Figures 2B and 2C). These in vitro data, therefore, do not support a contribution of ticagrelor bacteriostatic or bactericidal activity to the in vivo observed prevention of IE.

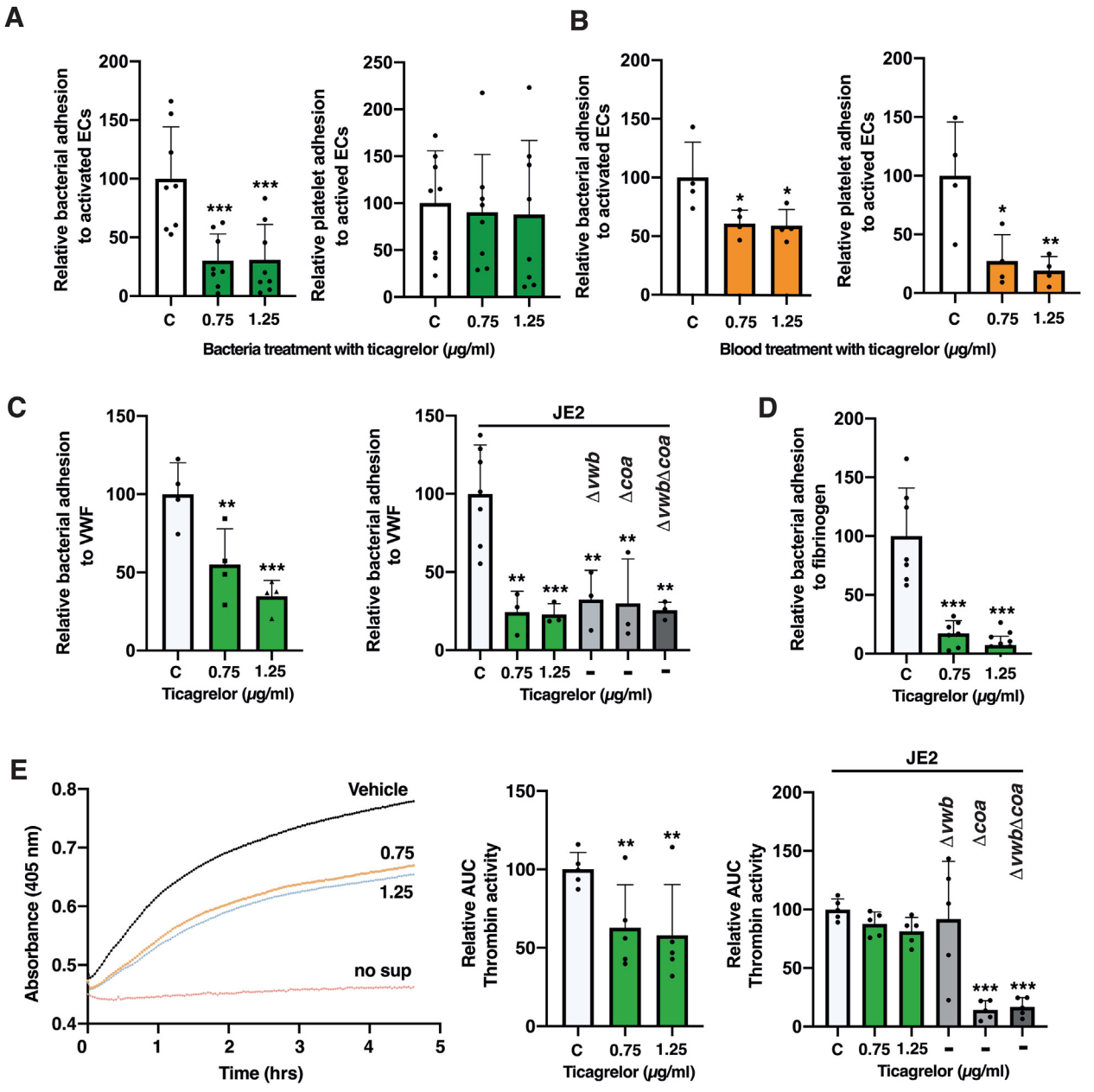
CONVENTIONAL CLINICAL DOSAGE OF TICAGRELOR INHIBITS MASTER REGULATORS OF *S AUREUS* VIRULENCE. We assessed whether ticagrelor could alter the expression of key virulence factors by *S aureus*. In Western blotting experiments, we observed a reduced α-toxin production in supernatants of IE clinical isolate cultures grown in the presence of ticagrelor (0.75 mg/mL) as compared with vehicle (Figure 3A). Accordingly, *S aureus* supernatant hemolytic activity was substantially inhibited by ticagrelor (Figure 3B). This inhibition of hemolytic activity was observed for both the clinical isolate and the JE2 strain at 1.25 mg/mL ticagrelor concentration (Figure 3B). The residual hemolytic activity of ΔHla (Figure 3A) was also inhibited by ticagrelor (Supplemental Figure 6), which further depicted an effect of this drug on the production of other bacterial pore-forming toxins. We found that ticagrelor-treated JE2 bacteria produced lower levels of PVL, a typical MRSA virulence factor, than control bacteria (Supplemental Figure 6). The production of the carotenoid pigment staphyloxanthin[14] was also significantly hampered by ticagrelor, as evidenced by measuring the absorbance of bacterial lysates and by visual loss of typical *S aureus* golden color (Figure 3C). We then investigated the ability of ticagrelor to induce changes in mRNA expression of major regulators of *S aureus* virulence.[15] Quantitative reverse transcriptase polymerase chain reaction analysis revealed that a 6-hour treatment of the IE clinical isolate with ticagrelor (1.25 mg/mL) led to the downregulation of the quorum-sensing accessory gene regulator (Agr) system components, including *agrA* and *agrC*, as well as downstream RNAIII, *hla* and *psma* effectors, of the histidine kinase SaeR encoded by the *sae* (*S aureus* exoprotein) locus and of *mgrA* (HTH-type transcriptional regulator) (Figure 3D). In contrast, ticagrelor did not modify mRNA expression of the other staphylococcal accessory regulator A. Thus, these data indicate that conventional clinical dosage of ticagrelor alters Agr- and SaeRS-regulated *S aureus* virulence, resulting in the inhibition of toxin production.

TICAGRELOR PREVENTS *S AUREUS* ADHESION ON THE ECM AND ACTIVATED ENDOTHELIAL CELLS. We next assessed the effect of ticagrelor on bacteria adhesion to activated endothelial cells (EC) under flow. HUVECs were activated with tumor necrosis factor-α, which induces the release of cell-bound VWF. Labelled bacteria were added to human blood before perfusion over the activated EC surface at a shear rate of 1,000 s^{-1}. Pretreating bacteria with ticagrelor (0.75-1.25 mg/mL) strongly impaired their ability to adhere to EC (Figure 4A, left) even though this did not diminish platelet adhesion (Figure 4A, right). In contrast, treating blood with ticagrelor before perfusion inhibited both bacteria and platelet adhesion to activated EC (Figure 4B). To study the impact of bacteria treatment by ticagrelor on bacteria interaction with VWF independent of plasma proteins and platelets, bacteria suspensions were perfused over VWF-coated surfaces. The adhesion of both the IE clinical isolate and JE2 strain to VWF was hampered by ticagrelor to the same extent as vWbp or Coa deficiency in the JE2 background (Figure 4C). We also found that pretreating the clinical strain by ticagrelor impaired bacterial adhesion to surface-bound fibrinogen (Figure 4D), whereas plasma-induced clumping of the *S aureus* clinical isolate, mainly caused by binding soluble fibrinogen to bacterial clumping factors, was not prevented by ticagrelor (Supplemental Figure 7). To study the effect of ticagrelor on *S aureus* coagulase activity,[16] we analyzed prothrombin activation in human plasma after addition of supernatants from bacteria pretreated with ticagrelor or vehicle. Coagulase activity in bacterial supernatants was decreased by ticagrelor for the IE clinical strain (Figure 4E) but not for the JE2 strain (Figure 4E). In our assay, Coa, but not vWbp, was necessary for coagulase activity of JE2, as shown by use of isogenic Coa- (Δ*coa*) and vWbp-deficient

FIGURE 3 Continued

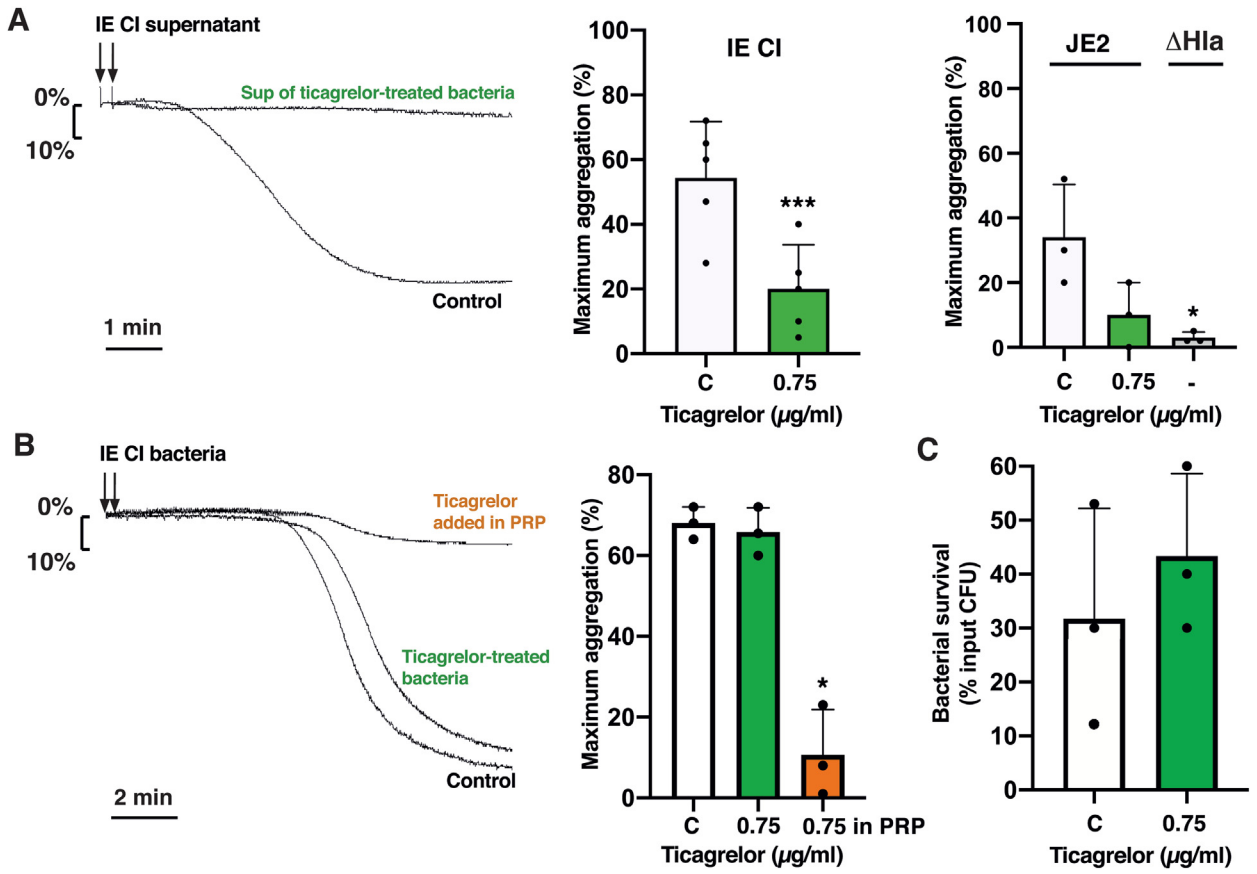
JE2, JE2 ΔHla, and IE CI bacteria grown in the presence of indicated concentrations of ticagrelor or vehicle (**C**: 1% DMSO). **(A)** Western blot detection of α-toxin in bacterial supernatants. Data represent median and 25th-75th percentiles (n = 8; *$P < 0.05$; ***$P < 0.001$, Kruskal-Wallis and Dunn's multiple comparison tests). Vehicle data are arbitrarily set to 100. **(B)** Hemolytic activity of bacterial supernatants (IE CI, n = 5: *$P < 0.05$, **$P < 0.01$; JE2, n = 4: *$P < 0.05$, **$P < 0.01$). **(C)** Pigment intensity in bacterial pellets (IE CI, n = 7; JE2, n = 6; ***$P <$ 0.001). Data in **C** and **D** are the mean ± SD (*P*: ticagrelor vs vehicle, analysis of variance and Dunnett's multiple comparison test). **(D)** Quantitative reverse transcriptase polymerase chain reaction analysis of messenger RNA expression in exponentially phase IE CI (n = 4, *$P <$ 0.05, *$P < 0.05$, ***$P < 0.001$ vs vehicle, Student's *t*-test). Data represent the mean ± SD. Abbreviations as in Figure 1.

FIGURE 4 Conventional Concentrations of Ticagrelor Alter *S aureus* Adhesive Properties



(A) Human whole blood perfused with bacteria over activated HUVEC. Bacteria were pretreated with ticagrelor or vehicle (vehicle, n = 8; 0.75 mg/mL, n = 8; 1.25 mg/mL, n = 8; $^{**}P < 0.01$). **(B)** Ticagrelor or vehicle was added to blood just prior perfusion (vehicle, n = 4; 0.75 mg/mL, n = 4; 1.25 mg/mL, n = 4; $^{*}P < 0.05$, $^{**}P < 0.01$). **(C)** Bacteria suspensions perfused over VWF (IE CI: vehicle, n = 4; 0.75 mg/mL, n = 4; 1.25 mg/mL, n = 4; JE2: vehicle, n = 7; 0.75 mg/mL, n = 3; 1.25 mg/mL, n = 3; JE2 Δ*coa*, Δ*vwb*, and Δ*coa*Δ*vwb*: n = 3; $^{**}P < 0.01$, $^{***}P < 0.001$). **(D)** Bacteria adhesion on fibrinogen (vehicle, n = 7; 0.75 mg/mL, n = 7; 1.25 mg/mL, n = 7; $^{**}P < 0.01$, $^{***}P < 0.001$). **(E)** Thrombin activity in bacterial supernatants added to human plasma (IE CI: n = 5, $^{**}P < 0.01$; JE2: n = 5, $^{***}P < 0.01$). All data represent the means ± SD (*P*: ticagrelor vs vehicle, analysis of variance, and Dunnett's multiple comparison test). Vehicle control is arbitrarily set to 100. EC = endothelial cells; HUVEC = human umbilical vein endothelial cells; VWF = von Willebrand factor; other abbreviations as in Figure 1.

FIGURE 5 Effect of Ticagrelor Treatment on Toxin-Induced Platelet Aggregation



(A) Platelet aggregation in human PRP was induced by supernatants of bacteria that have been pretreated with ticagrelor or vehicle (control). Representative aggregation curves are shown on the **left**. **(Middle)** n = 5, ***$P < 0.001$, 2-tailed paired *t*-test. **(Right)** n = 3, *$P < 0.05$ vs vehicle; analysis of variance and Dunnett's multiple comparison test. **(B)** Platelet aggregation was induced by bacterial cells that have been pretreated with ticagrelor or vehicle (control). Alternatively, ticagrelor was added in PRP before bacteria addition. Representative aggregation curves are shown **(left)**. **(Middle)** n = 3, *$P < 0.05$ vs vehicle, analysis of variance, and Dunnett's multiple comparison test. **(Right)** n = 3, $P > 0.05$, 2-tailed paired *t*-test. **(C)** Platelet-killing assay performed with human washed platelets and IE IC. Bacterial survival is plotted as percentage of initial CFU input (n = 3, $P > 0.05$, 2-tailed paired *t*-test). All data represent the mean ± SD. Abbreviations as in Figure 1.

bacteria (Δ*vwb*) (Figure 4F). Ticagrelor, thus, inhibits bacterial interaction with activated EC via multiple mechanisms.

TICAGRELOR LIMITS THE ABILITY OF *S AUREUS* TO INDUCE PLATELET AGGREGATION VIA SECRETED TOXINS. To study the impact of ticagrelor on *S aureus* interaction with platelets,[17] we performed platelet aggregation assays in human PRP. As previously reported,[18] we observed that supernatants from ΔHla JE2 bacteria failed to induce platelet aggregation (Figure 5A). In agreement with our data showing decreased production of α-toxin by ticagrelor-pretreated IE clinical isolate, we found that supernatants from bacteria that have been grown in the presence of ticagrelor provoked less platelet aggregation as compared with control supernatants (Figure 5A). In contrast, adding ticagrelor to PRP before addition of bacterial supernatant did not prevent platelet aggregation (Supplemental Figure 8).

Next, we conducted platelet aggregation assays using bacterial cells.[19] Whereas addition of ticagrelor to PRP before the assay potently inhibited platelet responses to bacteria (Figure 5B), pretreating the IE clinical isolate with ticagrelor neither delayed nor impaired bacteria-induced platelet aggregation. Therefore, ticagrelor interferes with the ability of toxins secreted by *S aureus* to initiate platelet aggregation, whilst it does not prevent platelet-bacteria bridging by soluble plasma proteins and subsequent platelet aggregation, independently of its antiplatelet

activity. Importantly, in a platelet-killing assay, platelets killed ticagrelor-treated *S aureus* clinical isolate as efficiently as control bacteria (**Figure 5C**).

DISCUSSION

Our study provides the first experimental evidence that clinically relevant concentrations of ticagrelor can prevent IE and identifies a unique virulence factor–inhibiting effect of ticagrelor as the underlying mechanism. Using a previously established mouse model of *S aureus* IE, administration of ticagrelor at antiplatelet dosage before infection could prevent the formation of infected vegetations. We further demonstrated that the clinical dosage of ticagrelor possesses platelet-independent antibacterial activity through its ability to inhibit toxin production and bacterial adherence, 2 key determinants of *S aureus* virulence. In mice, the P2Y12 inhibitor clopidogrel failed to prevent IE development. IE prevention by ticagrelor could, hence, at least partly, be due to the antivirulence activity against *S aureus* reported here. In terms of clinical perspectives, because ticagrelor was given before infection in our in vivo model, our data are more appropriate in terms of IE prophylaxis. Dual antiplatelet therapy (DAPT) with aspirin and a P2Y12 receptor inhibitor is the treatment of choice for the prevention of atherothrombotic events in patients with acute coronary syndromes (ACS). Patients hospitalized with ACS, especially those with comorbid conditions, are at a considerable risk for infectious complications. In the study by Lupu et al,[20] it was shown that patients treated with DAPT that includes ticagrelor have a 64% lower risk of gram-positive infection during the first year after hospitalization compared with patients treated with DAPT treatment that includes clopidogrel. DAPT is also recommended in patients with bioprosthetic valve implantation and recent coronary stenting. In contrast, aspirin alone has emerged in recent consensus statement to treat patients receiving transcatheter aortic valve implantation (TAVI),[21] although not fully implemented in daily clinical practice. Hitherto, ticagrelor is not indicated in these patients, but we are waiting for the results of some ongoing registries. Ticagrelor monotherapy does not increase the risk of bleeding as compared with aspirin alone,[22] but it decreased bleeding significantly without increasing ischemic events compared with ticagrelor plus aspirin in patients with ACS.[23] Hence, owing to its potent antibacterial effect and the increased risk of infection in patients receiving bioprosthetic heart valve, there may be a potential benefit of using ticagrelor for these patients.

In view of a role for platelets in the initial steps leading to vegetation formation, including bacterial capture on the surface of activated EC, several studies have been performed to assess the benefits of antiplatelet drugs in IE. However, although data from in vitro and in vivo preclinical studies showed decreased vegetation formation with aspirin, clinical studies in patients receiving medically indicated aspirin therapy were not conclusive, and the clinical usefulness of antiplatelet approaches in IE has been questioned.[24] To date, no studies ever investigated whether prior ticagrelor or clopidogrel therapy could prevent IE.

We present 2 possible mechanistic explanations of IE prevention by ticagrelor. The first mechanism relies on the ability of ticagrelor to inhibit bacterial adhesion to activated EC. We showed that bacteria that have been grown in the presence of sub-minimal inhibitory concentration ticagrelor, corresponding with its antiplatelet dose, had impaired adhesion to activated EC in flowing blood, which fairly mimics the inflamed aortic valve surface. By performing bacterial adhesion assays without blood components, we further showed that ticagrelor treatment led to altered bacterial binding to VWF and fibrinogen. In the presently used mouse model of inflammation-related IE, platelet depletion could prevent bacterial adhesion to inflamed valves, which led to the proposition that platelets could play a key role at the early phase of IE by capturing bacteria on VWF-exposing EC. In our study, platelet inhibition with clopidogrel neither prevented IE nor decreased bacterial content in vegetations. It is, therefore, unlikely that ticagrelor antiplatelet activity solely explains IE prevention in mice. In contrast, these data support a role for the direct antibacterial effect of ticagrelor in preventing IE. Our observation that supernatants from ticagrelor-treated bacteria had diminished coagulase activity could also explain decreased *S aureus* interaction with valvular endothelia. Indeed, it has been suggested that staphylothrombin-generated fibrin could be involved in the initiation of vegetation development by promoting platelet-bacteria interactions.[19] With regard to the potential usefulness of other antiplatelet approaches in IE, it could still be interesting to study the effect of drug compounds that target the mechanisms of platelet adhesion on the endothelium, such as platelet GPIb or VWF.[25]

The molecular explanation of ticagrelor-mediated loss of bacterial adherence remains unknown. Bacterial adhesion to endothelia depends on interaction of bacteria with host molecules acting as anchoring points, including vWbp binding to VWF, and the adhesins, ClfA, FnBPA, and FnBPB interacting with

fibrinogen or fibronectin. ClfA is crucial for *S aureus* clumping by binding both ends of fibrinogen. Clumping is thought to be a prerequisite for bacterial adhesion and the formation of fibrin-coated aggregates. However, using an in vitro clumping assay, we could not prevent clumping of the IE *S aureus* clinical isolate by ticagrelor, whereas adhesion to surface-bound fibrinogen was strongly impaired. FnBPA, but not ClfA, has been reported to mediate EC activation in an in vitro model of IE.[26] Accordingly, ClfA-deficient mutants were not impaired in their ability to induce inflammation-dependent IE in mice.[11] However, in the same model, fibrinogen depletion with ancrod had no effect on vegetation formation, and the mechanisms of bacterial adhesion to the inflamed valve could not be elucidated. Because we observed a downregulation of MgrA mRNA expression in ticagrelor-treated bacteria, the loss of *S aureus* adhesive properties may be related to reduced ArlRS-MgrA signaling-dependent repression of giant inhibitory surface proteins.[27] Indeed, it has been shown that deficiency of ArlRS-MgrA in *S aureus* results in impaired bacterial adhesion to damaged vessels in mice.[27]

The second mechanism relates to the downregulation of the *S aureus* Agr system by ticagrelor and inhibition of toxin production. We found that growing bacteria in the presence of ticagrelor resulted in a drastic inhibition of genes regulated by Agr, a quorum-sensing system and master regulator of *S aureus* virulence, and most particularly of its effector RNAIII, which plays a central role in toxin production.[28] In a rabbit model of endocarditis, RNAIII expression was shown to be progressively increased in vegetations along with bacterial densities.[29] However, contradictory clinical observations have been reported regarding the role of Agr during endovascular infection.[30] Our observation that ticagrelor inhibits RNAIII and downstream α-toxin mRNA expression is consistent with decreased production of this toxin in supernatants from stationary phase bacteria that have been grown in the presence of this drug. Importantly, in mice, α-toxin infusion promotes bacterial adhesion to inflamed valve by activating EC.[11] A recent study indicated that the presence of small amounts of α-toxin in blood abrogates thrombus formation.[31] Thus, limiting α-toxin production to subcytolytic levels might inhibit platelet-dependent vegetation formation. In contrast, at cytolytic concentrations, α-toxin causes aberrant platelet activation and aggregation, as a bacterial immune evasion mechanism. Notwithstanding, in a mouse model of staphylococcal sepsis, α-toxin neutralization could prevent microvascular dysfunction and thrombosis while not interfering with beneficial antimicrobial platelet responses.[18] Recently, it has been reported that α-toxin was able to accelerate platelet clearance by the hepatic Ashwell-Morell receptor through the release of endogenous platelet sialidase, leading to thrombocytopenia during *S aureus* bacteraemia.[8] Ulloa et al[5] also showed that ticagrelor could prevent α-toxin-induced inhibition of platelet antibacterial activity. Indeed, in vitro, platelet pretreatment with ticagrelor improved *S aureus* killing. Conversely, we show that ticagrelor-treated bacteria, which produced less α-toxin, were as efficiently killed by platelets as control bacteria. Therefore, the inhibition of α-toxin production by ticagrelor could contribute to preserved platelet antimicrobial function, whereas ticagrelor antiplatelet activity could improve platelet-killing ability. In agreement with previous reports,[32] we also observed potent inhibition of bacteria-induced platelet aggregation by ticagrelor, which might contribute to limited vegetation growth on ticagrelor therapy. Of note, in a recent study performed on 34 IE CI, no correlation could be found between platelet-bacteria interactions in PRP and disease severity.[33]

STUDY LIMITATIONS. One can wonder whether a single dose of ticagrelor may affect *S aureus* pathogenicity *in vivo* ≤3 days later. In the presently used mouse model of IE, it has previously been shown that bacteria adhesion to the valve occurs almost immediately (within minutes) after catheter insertion and histamine infusion.[11] Moreover, since histamine has a very short half-life in vivo (several minutes),[34] the inflammation-triggered bacteria adhesion is limited in time, and mice that do not show bacteria on their valve immediately after infusion will not develop endocarditis 3 days later. Therefore, the observed inhibition of bacterial adhesion to inflamed EC in our in vitro experimental setting in which bacteria were grown in the presence of ticagrelor before being washed and used in the adhesion assays fairly reproduces what happens in vivo when bacteria are injected intravenously after ticagrelor administration. Bacteria circulating in the bloodstream in the presence of ticagrelor will have a decreased ability to adhere to the valve after histamine infusion. In contrast, we do not have in vitro experimental evidence showing that a single dose of ticagrelor can inhibit the agr system and subsequent toxin production up to 3 days in vivo. The in vivo monitoring of agr activity in mice receiving ticagrelor or vehicle could provide an answer to this question, but this is hardly possible owing to detection limits of currently available noninvasive imaging techniques. This is a

limitation of our study. Also, the question as to know whether sustained platelet inhibition by ticagrelor, for example, over 3 days in our mouse models, would improve IE prevention could not be addressed in the present study and remains an open question for future investigation. Our study did not evaluate the effect of ticagrelor as a treatment of IE either. Of interest, a recent study in mice showed that the administration of ticagrelor after infection could decreased prosthetic joint infection with *S aureus*.[35]

Ticagrelor diminished bacterial toxin production and adherence irrespective of the *S aureus* strain background used. Despite the fact that no clear relationship between virulence profiles of IE CIs could be deciphered so far,[33] the ability of ticagrelor to inhibit bacterial signaling systems, particularly quorum sensing,[36] may be highly relevant to IE where antibiotic resistance is increasingly problematic.[37]

CONCLUSIONS

In summary, we report here that a clinically relevant dosage of ticagrelor prevents the development of IE in a mouse model of inflammation-induced IE induced by a clinical *S aureus* isolate. Our in vitro findings indicate that ticagrelor directly impacts bacterial pathogenicity by altering bacterial adhesive properties and global virulence factor expression, while not interfering with platelet killing capacity, which may contribute to the ability of the drug to prevent IE.

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PERSPECTIVES

COMPETENCE IN MEDICAL KNOWLEDGE: IE remains a deadly disease with a mortality rate of approximately 20% to 40%. The main pathogens responsible for IE are gram-positive bacteria, with *S aureus* being the most frequent and virulent. Current antibiotic-based approaches against IE lack efficacy. Finding novel means to adequately prevent this disease is, therefore, of paramount importance. Our results in mice provide strong evidence to support the fact that, in the antiplatelet therapeutic armory, ticagrelor could confer antibacterial protection compared with other antiplatelet drugs. In the presence of human blood, we found that the typical clinical dosage of ticagrelor possesses antivirulence activity against *S aureus*. Ticagrelor makes *S aureus* less virulent by inducing major changes in the bacterial quorum sensing system and adherence, which represents a novel mode of action as compared with any currently available antibiotics.

TRANSLATIONAL OUTLOOK: These findings may motivate new initiatives for the design of randomized clinical trials aimed at studying protective effect of ticagrelor adjuvant therapy against Gram-positive bacterial infection after surgical or transcatheter valve implantation as compared with conventional preventive strategies. As an antivirulence agent, ticagrelor would not likely impose a high selective pressure on bacteria, therefore limiting the development of resistance and dissemination of virulence genes.

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KEY WORDS bacterial virulence, infective endocarditis, *Staphylococcus aureus*, ticagrelor

APPENDIX For an expanded Methods section as well as a supplemental table and figures, please see the online version of this paper.