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Platelet Deficiency Represents a Modifiable Risk Factor for Periprosthetic Joint Infection in a Preclinical Mouse Model

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Abstract

Background: Well known for their hemostatic function, platelets are increasingly becoming recognized as important immunomodulators. The purpose of the present study was to assess the impact of platelet depletion on antimicrobial host defense in a mouse model of periprosthetic joint infection (PJI).

Methods: Thrombocytopenia (TCP) was induced in C57BL/6 mice with use of a selective antibody against platelet CD41 (anti-CD41). Whole blood from pre-treated mice was incubated with *Staphylococcus aureus* to assess antimicrobial efficacy with use of bioluminescent imaging, quantitative histological staining, and colony forming unit (CFU) quantification. In parallel, untreated heterologous platelets were added to TCP blood to assess potential rescue of antimicrobial efficacy. In vivo, TCP and control mice underwent placement of a titanium implant in the femur inoculated with bioluminescent Xen36 *S. aureus*. Longitudinal bioluminescent imaging was performed postoperatively to quantify the evolution of bacterial burden, which was confirmed via assessment of *S. aureus* CFUs on the implant and in peri-implant tissue on postoperative day (POD) 28.

Results: Anti-CD41 treatment resulted in significant dose-dependent reductions in platelet count. Ex vivo, platelet-depleted whole blood demonstrated significantly less bacterial reduction than control blood. These outcomes were reversed with the addition of untreated rescue platelets. In vivo, infection burden was significantly higher in TCP mice and was inversely correlated with preoperative platelet count ($r^2 = 0.63$, $p = 0.037$). Likewise, CFU quantification on POD28 was associated with increased bacterial proliferation and severity of periprosthetic infection in TCP mice compared with controls.

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Conclusions: Thrombocytopenia resulted in an increased bacterial burden both ex vivo and in vivo in a mouse model of PJI.

Clinical Relevance: In orthopaedic patients, deficiencies in platelet quantity or function represent an easily modifiable risk factor for PJI.

Periprosthetic joint infection (PJI) is a devastating complication of arthroplasty, often necessitating multiple reoperations and prolonged treatment with systemic antibiotics[1]. Because of the tremendous negative impact on health and quality of life associated with PJI, attention has increasingly focused on innovative approaches for prevention [2–8]. Despite decades of attempts to optimize antimicrobial prophylaxis, implant sterility, and other exogenous factors, the incidence of revision surgery for PJI has continued to rise[9,10]. As a result, there has been a shift in focus in recent years toward enhancing the host immune response. While medical comorbidities such as diabetes mellitus and obesity are associated with a heightened risk of PJI, modifying these factors requires considerable and prolonged efforts[11–13]. As such, the identification of endogenous host factors that can be perioperatively modified remains paramount.

Thrombocytopenia (TCP) is a modifiable factor that occurs in 5% to 10% of patients undergoing surgery[14–22].While the hemostatic role of platelets is well established, the role that platelets play in the host immune response is less widely appreciated. Platelets readily detect microbial pathogens, contain granules filled with antimicrobial peptides, recruit and activate immune cells, and trap and internalize pathogens to promote clearance[23–29]. In addition, platelets act synergistically with antibiotics to enhance pathogen eradication[30]. Platelets are particularly appealing as a tool for infection prevention as they migrate to sites of tissue damage—precisely where infective pathogens are most likely to infiltrate. TCP thus represents a logical and promising modifiable potential risk factor for PJI.

Despite the recent mechanistic studies identifying the antimicrobial functions of platelets, the clinical magnitude remains unknown. Multiple studies have demonstrated that TCP in surgical patients portends increased risks of death and major postoperative complications, including infection[17,31–35]. However, causation cannot be concluded, as TCP is generally accompanied by systemic illness rendering patients more susceptible to infection as well as postoperative hematoma, a known risk factor for PJI[36–39]. As such, the objective of the present study was to determine the mechanistic relationship between platelet count and infectious burden and to assess the modifiability of this factor. We hypothesized (1) that platelet depletion would result in an increased rate and severity of PJI in a well-validated mouse model and (2) that restoration of normal platelet quantity would reconstitute antimicrobial host defense.

Materials and Methods

Statement of Ethics

All animal procedures were approved by the institution’s Animal Research Committee (ARC#2008-112-41).

Mice

Twelve-week-old, 20 to 25-g C57BL/6 wild-type mice (Jackson Laboratory) were used for all experiments. All mice were housed 4 per cage with a 12-hour light and dark cycle, free access to water, and a standard pellet diet. Institutional veterinary staff evaluated their daily health throughout the experiment.

Induction of Thrombocytopenia

Mice were randomized to treatment with a retro-orbital injection of anti-CD41 (purified anti-mouse CD41 immunoglobulin G [IgG] antibody targeting the α-integrin IIb subunit expressed on platelets; BioLegend) or inactive isotype control IgG (purified rat; BioLegend). This inactive antibody has no effect on mouse platelet count or host immunity and was employed in order to ensure that differences between the experimental and control groups were specifically due to anti-CD41 and not secondary to the injection itself[40,41]. Two different doses of anti-CD41 were utilized in order to simulate moderate (0.4 μg/g) or severe (2.0 μg/g) TCP in vivo. Control antibody was administered at a dose of 0.4 μg/g. All antibody preparations were made via dilution in phosphate-buffered saline solution (PBS), pH 7.2, to a volume of 100 μL per injection. For in vivo experiments, mice were injected 24 hours prior to surgery. On the basis of studies demonstrating a recovery in platelet count 24 to 72 hours after initial injection, a second injection was administered 72 hours after the first in order to prolong the duration of platelet depletion[40,41]. To confirm TCP, blood was collected from the retro-orbital plexus of mice from each group immediately prior to surgery (24 hours after injection) and again on postoperative day 2 (POD2, 72 hours after injection). Cell counts were performed with use of a hemocytometer (Genesis Diagnostics).

Staphylococcus aureus Bioluminescent Strain

Staphylococcus aureus Xen36 (PerkinElmer) is a modified strain containing a bioluminescent *lux* operon stably integrated by plasmid homologous recombination. A blue-green luminescent signal is expressed by viable, metabolically active *S. aureus*[42]. The validity and consistency of the bioluminescent signal has been confirmed in previous studies[43–46]. Bacteria were grown and cultured as previously described[43–46].

Ex Vivo Assessment

Preparation of Rescue Platelets—Fresh whole blood was collected via cardiac puncture from 4 untreated and uninfected C57BL/6 wild-type mice and was pooled into polypropylene tubes containing sodium citrate as an anticoagulant (1:5 [vol/vol]). Blood was then centrifuged (400 ×g for 20 minutes at 20°C) to yield an upper platelet-rich-plasma suspension. The upper two-thirds of this suspension layer was transferred to fresh polypropylene tubes and was centrifuged (800 ×g for 10 minutes at 20°C) to yield platelet pellets. Pellets were resuspended in 200 μL of PBS, pH 7.2[47].

Blood Preparation and Culture—Different mice were utilized for ex vivo and in vivo experiments. Twenty-four hours following anti-CD41 treatment, mice were anesthetized via inhalation of isoflurane (2%). Whole blood was retrieved via cardiac puncture from 4 mice per treatment group and pooled into heparin-coated collecting tubes (Sigma Aldrich)

containing EDTA (ethylenediaminetetraacetic acid, 2%, 1:10 [vol/vol]; Sigma Aldrich). Blood was inoculated with *S. aureus* Xen36 (1:1 [vol/vol]) to achieve a 10^6 colony forming unit (CFU)/mL inoculum. The resulting whole blood cultures were incubated at 37°C for 24 hours in 96-well plates (200 μL/well). In order to assess reversibility, platelets were prepared as above and added to relevant treatment groups after 1 hour of culture.

Ex Vivo Quantification of *S. aureus* Viability and Biofilm Formation—To evaluate the effect of TCP on viability of *S. aureus* Xen36 in whole blood cultures, bioluminescence imaging with use of the IVIS Lumina X5 imaging system (PerkinElmer) was performed 3 hours post-inoculation[44–46,48]. Data were quantified as total flux (photons/s/cm^2/sr). The relative burden of *S. aureus* biofilm after exposure to differing levels of TCP whole blood was evaluated after 24 hours of incubation with use of an established crystal violet assay protocol[49]. Absorbance was measured at 585 nm with use of a spectrophotometer (FLUOstar Omega; BMG Labtech). Finally, the *S. aureus* Xen36 was diluted in tryptic soy broth (TSB) to a concentration of 10^3 CFU/mL, combined with whole blood of pretreated mice in polypropylene tubes (1:1 [vol/vol]) and incubated for 45 minutes at 37°C. The inoculated blood culture was quantitively plated onto TSB agar (20 μL/plate). Total CFUs were counted after 16-hour incubation at 37°C and were expressed as CFU/mL.

In Vivo Assessment

Surgical Procedure—Mice were anesthetized via inhalation of isoflurane (2%). As previously described, a titanium Kirschner wire (0.8 mm in diameter, 6 mm in length; DePuy Synthes) was placed retrograde into the distal part of the femur, and the joint was inoculated with 1×10^3 CFUs of *S. aureus* Xen36 in 2 mL of PBS or with 2 mL of sterile saline solution (0.9% NaCl)[43,45,48].

In Vivo Quantification of *S. aureus* Infectious Burden—Staphylococcal burden over time was tracked via bioluminescence imaging as previously described and was quantified as total flux (photons/s/cm^2/sr) with use of Living Image software (PerkinElmer)[43–45,48].

After the animals were killed on POD28, implants were removed and bacteria were detached via sonication in 500 μL of 0.3% Tween-polysorbate 80 in TSB for 15 minutes. Surrounding tissue was prepared by homogenizing harvested bone and joint tissue (Pro200H Series homogenizer; PRO Scientific). Bacterial CFUs from implant and tissue were quantified as CFU/mL after overnight culture on agar plates.

Statistical Analysis

For in vivo experiments, 44 mice were included for analysis (inactive antibody, n = 12; low-dose anti-platelet antibody, n = 14; high-dose anti-platelet antibody, n = 14; sterile control, n = 4) on the basis of previous literature demonstrating that at least 6 mice per group are necessary to demonstrate significance at the $p < 0.05$ level[45]. Blood for cell-count analysis was obtained from 4 mice from each group. Ex vivo 96-well plate experiments were repeated twice independently on different days, and each experiment included 8 wells per treatment or control group (n = 16 wells/group). Four plates were included per group for ex vivo CFU analysis. Data were compared with use of 2-tailed Student t test or Fisher exact

test, as appropriate. All data were expressed as the mean and the standard error of the mean (SEM). The level of significance was set at $p < 0.05$. Linear regression analysis was utilized to assess for correlation between platelet count and infectious burden.

Results

Induction of Thrombocytopenia

Twenty-four hours after administration of anti-platelet antibody, the preoperative mean platelet count was successfully reduced in both the low-dose (LD) and high-dose (HD) groups compared with the inactive antibody (IA) controls ($267.3 \pm 55.6 \times\times 10^3/\mu L$, $153.0 \pm 22.5 \times 10^3/\mu L$, and $671.6 \pm 129.6 \times 10^3/\mu L$, respectively). These reductions exhibited a dose-dependent response ($p = 0.009$ and $p < 0.001$ for the IA group versus the LD and HD groups, respectively) (Fig. 1-A). After 72 hours (48 hours postoperatively), the mean platelet count remained significantly lower in the LD group compared with the IA group ($413.2 \pm 51.6 \times 10^3/\mu L$ versus $904.5 \pm 155.4 \times 10^3/\mu L$, $p = 0.007$) and in the HD group compared with both the IA and LD groups ($278.3 \pm 32.5 \times 10^3/\mu L$ versus $904.5 \pm 155.4 \times 10^3/\mu L$ [$p < 0.001$] and $278.3 \pm 32.5 \times 10^3/\mu L$ versus $413.2 \pm 51.6 \times 10^3/\mu L$ [$p = 0.038$], respectively).

There was no significant difference in hemoglobin between the 3 groups preoperatively or postoperatively (Fig. 1-B). Additionally, the groups exhibited similar white blood-cell (WBC) count both preoperatively and postoperatively (Fig. 1-C).

Influence of Thrombocytopenia on Bacterial Burden ex Vivo

Ex vivo bacterial bioluminescent signal was significantly higher in the LD and HD groups compared with the IA group ($5.2 \times 10^6 \pm 5.1 \times 10^5$ versus $3.1 \times 10^6 \pm 2.7 \times 10^5$ photons/s/cm^2/sr [$p < 0.001$] and $6.4 \times 10^6 \pm 1.9 \times 10^5$ versus $3.1 \times 10^6 \pm 2.7 \times 10^5$ photons/s/cm^2/sr [$p < 0.001$], respectively) (Fig. 2-A). Additionally, bioluminescence was significantly higher in the HD group compared with the LD group ($6.4 \times 10^6 \pm 1.9 \times 10^5$ versus $5.2 \times 10^6 \pm 5.1 \times 10^5$ photons/s/cm^2/sr, $p = 0.034$). When exogenous platelets were added to LD and HD cocultures, the TCP blood was successfully “rescued” and no significant difference in bacterial burden was observed when the LD and HD groups were compared with the IA group ($3.1 \times 10^6 \pm 3.9 \times 10^5$ versus $3.1 \times 10^6 \pm 2.7 \times 10^5$ photons/s/cm^2/sr [$p = 1.0$] and $3.0 \times 10^6 \pm 3.2 \times 10^5$ versus $3.1 \times 10^6 \pm 2.7 \times 10^5$ photons/s/cm^2/sr [$p = 0.813$], respectively).

The IA group demonstrated significantly lower bacterial biomass, as measured by absorbance, compared with the HD group (0.27 ± 0.05 versus 0.51 ± 0.07, $p = 0.046$) (Fig. 2-B). Absorbance was significantly reduced with the addition of rescue platelets in both the LD and HD groups (0.38 ± 0.09 to 0.08 ± 0.01 [$p = 0.003$] and 0.51 ± 0.07 to 0.24 ± 0.07 [$p = 0.012$], respectively). Addition of exogenous platelets reduced absorbance in the HD group to a level equivalent to the IA group (0.24 ± 0.07 versus 0.27 ± 0.05, $p = 0.731$) and in the LD group to a level significantly lower than the IA group (0.08 ± 0.01 versus 0.27 ± 0.05, $p = 0.001$).

After overnight culture, the HD group had a significantly higher average CFU count than the IA group ($3,900 \pm 670$ versus $1,900 \pm 370$ CFU/mL, $p = 0.016$) (Fig. 3). The LD

group demonstrated an average CFU count that fell between the IA and HD groups but was not significantly different from either group (2,530 ± 600 CFU/mL; p = 0.142 and p = 0.381 versus the HD and IA groups, respectively). Adding exogenous platelets to the HD cocultures significantly reduced the CFU count compared with the HD group alone (3,900 ± 670 versus 2,130 ± 440 CFU/mL, p = 0.038).

Influence of Thrombocytopenia on Bacterial Burden in Vivo

The IA, LD, and HD groups had similar bioluminescent signals on POD0 ($2.6 \times 10^4 \pm 2.7 \times 10^3$, $3.2 \times 10^4 \pm 3.7 \times 10^3$, and $2.4 \times 10^4 \pm 2.9 \times 10^3$ photons/s/cm^2/sr, respectively) and POD1 ($3.2 \times 10^5 \pm 6.8 \times 10^4$, $3.5 \times 10^5 \pm 1.6 \times 10^5$, and $2.7 \times 10^5 \pm 7.1 \times 10^4$ photons/s/cm^2/sr, respectively) (Figs. 4-A and 4-B). For the IA group, bioluminescent signal peaked on POD1 ($3.2 \times 10^5 \pm 6.8 \times 10^4$ photons/s/cm^2/sr), whereas the LD and HD groups peaked on POD3 ($4.6 \times 10^5 \pm 6.2 \times 10^4$ and $5.3 \times 10^5 \pm 1.8 \times 10^5$ photons/s/cm^2/sr, respectively). In the LD group, bioluminescent signal was significantly higher than that in the IA group at all time points after POD1 ($p < 0.05$ for all time points). In the HD group, bioluminescent signal was higher than in the IA group at all time points after POD1, although this was only significant at PODs 5, 7, 10, 18, 25, and 28 ($p < 0.05$). There was a significant negative correlation between mean bioluminescence across all time points and preoperative platelet count ($r^2 = 0.63$, p = 0.037) (Fig. 5).

On POD28, the mean CFU count was significantly higher in both the LD and HD groups compared with the IA group ($6.3 \times 10^5 \pm 1.6 \times 10^5$ versus $1.1 \times 10^5 \pm 7.2 \times 10^4$ CFU/mL [p = 0.007] and $1.1 \times 10^6 \pm 3.7 \times 10^5$ versus $1.1 \times 10^5 \pm 7.2 \times 10^4$ CFU/mL [p = 0.015], respectively) (Fig. 6). Viable *S. aureus* was found on 3 (25%) of 12 implants in the IA group versus 8 (61.5%) of 13 and 8 (72.7%) of 11 implants in the LD and HD groups, respectively (p = 0.111 and p = 0.039 for the IA group versus the LD and HD groups, respectively) (Table I).

Discussion

PJI remains a devastating complication, with the incidence continuing to rise despite targeted strategies of prevention and treatment[10]. Host factors, particularly those easily modified prior to surgery, have gained attention as a target of PJI prevention[48].Platelets possess functionality beyond their well-known role in coagulation, contributing meaningfully to host antimicrobial defense[23,24]. Indeed, the literature has emphasized a link between TCP and infection, although the clinical magnitude is difficult to discern[28,29]. In clinical studies, TCP has been associated with poor outcomes following infection, but causation is potentially confounded by the association of TCP with overall poor health status[17,31,33–35,50,51]. Additionally, TCP places patients at risk for postoperative hematoma formation, which is an independent risk factor for infection after surgery[36–39]. The present study, however, demonstrates that platelet depletion significantly increased infectious burden in a dose-dependent and reversible fashion. The current findings both validate the relationship between platelet count and infectious burden in a mouse model of PJI and establish direct causation on the basis of a series of mechanistic ex vivo experiments.

Additionally, the present study demonstrates the magnitude and the modifiability of this phenomenon.

In order to prevent excessive blood loss, the American Association of Blood Banks currently recommends prophylactic platelet transfusion for patients having major non-neuraxial surgery with platelets of $<50 \times 10^3$ cells/μL[52]. In the present study, even moderately thrombocytopenic mice experienced a significant increase in bacterial burden and implant infection, implying that even patients outside the transfusable range may be at increased risk for postoperative infection. Furthermore, the present study demonstrated that platelet deficiency at the time of surgery resulted in a sustained increase in infectious burden that persisted until the end of the 28-day experiment, despite a presumed recovery in platelet count after 48 hours[40,41]. Given that the effect of even a short-lived platelet deficiency was durable and long-lasting, early intervention in the perioperative period may be essential to prevent the life-changing repercussions of PJI.

The present study has several limitations. Utilizing a mouse model is a reproducible, easily modifiable, and cost-effective method to mechanistically investigate a hypothesis. However, the generalizability of results is limited and may not be applicable to larger animals and humans. Furthermore, TCP impairs hemostasis and is therefore a risk factor for postoperative hematoma, which could in turn impact infectious burden[36–39]. While qualitatively no appreciable hematoma was observed, hematoma formation in vivo was not explicitly monitored aside from noting no significant hemoglobin drop postoperatively. This limitation was addressed through mechanistic ex vivo studies, which corroborated an increased infectious burden in thrombocytopenic blood. Finally, while there is evidence that the inactive isotype control antibody has no effect on platelet count or host immunity, the impact of the control antibody was not directly assessed. Thus, there is a possibility that the antibody could have impacted bioburden, confounding the study results[40,41]. Despite these limitations, the current findings afford important significant results in multiple experimental models that underscore the impact of platelets in contributing to host defense against infection.

To our knowledge, the present study is the first to demonstrate that TCP results in increased bacterial burden both ex vivo and in vivo in a preclinical mouse model of PJI. TCP is easily identifiable, can be rapidly corrected, and represents a promising target for host-modification to prevent PJI. Clinical correlation in the surgical population is needed and should be a focus of further investigation.

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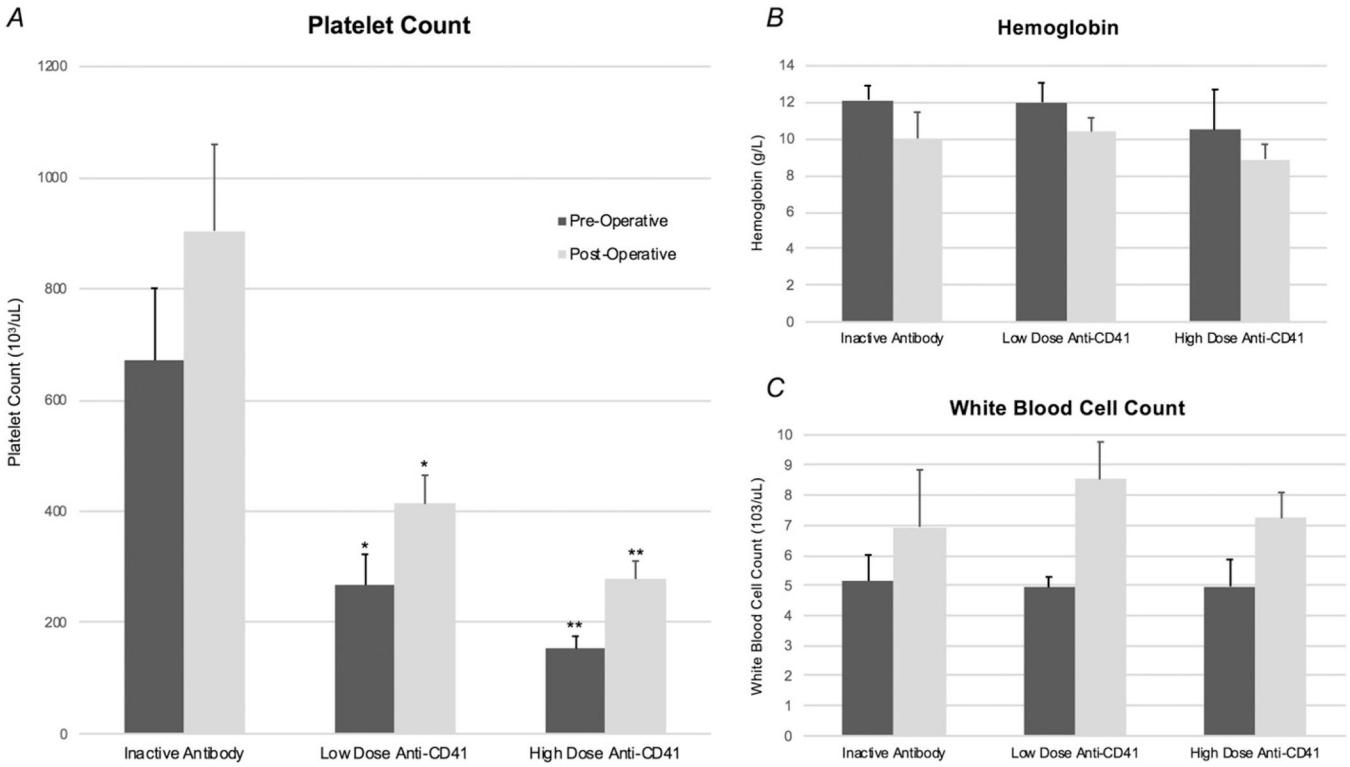

Fig. 1.

Figs. 1-A, 1-B, and 1-C Induction of thrombocytopenia using a platelet-specific antibody (n = 4 mice/group). Means and standard deviations are shown. *P < 0.05 versus inactive antibody group at corresponding time point. **P < 0.001 versus inactive antibody group at corresponding time point. Fig. 1-A Anti-CD41 effectively reduced mean platelet count in a dose-dependent fashion. Figs. 1-B and 1-C Anti-CD41 had no significant effect on mean hemoglobin (Fig. 1-B) or white blood-cell count (Fig. 1-C) at either time point.

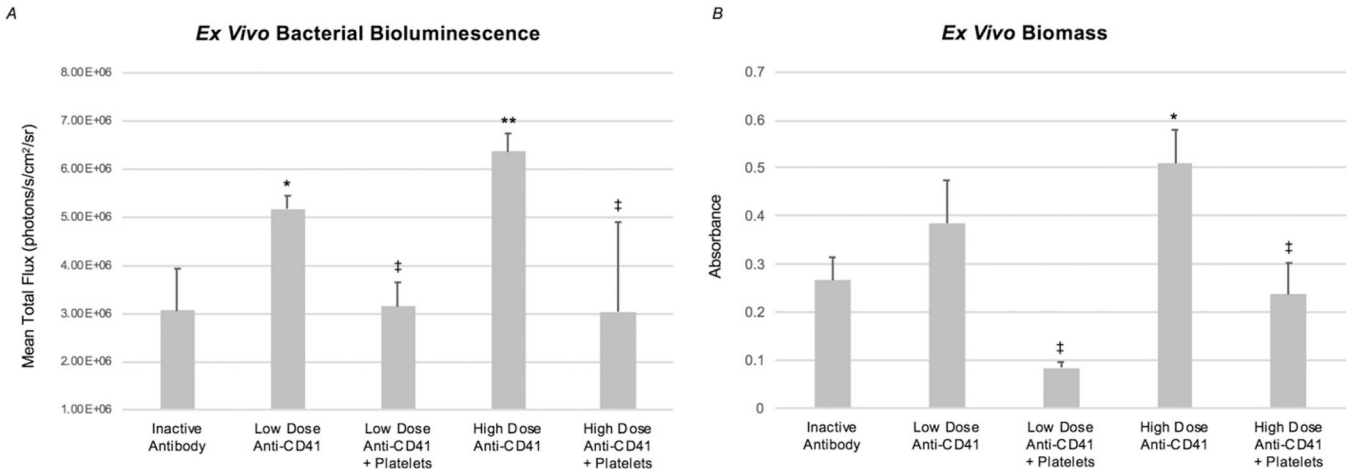

Fig. 2.

Figs. 2-A and 2-B Influence of thrombocytopenia on bacterial burden ex vivo platelets (n = 16 wells/group). *P < 0.05 versus inactive antibody group. Means and standard deviations are shown. **P < 0.05 versus both low-dose anti-CD41 and inactive antibody groups. ‡P < 0.05 versus corresponding group without the addition of exogenous. Fig. 2-A *S. aureus* activity, as measured by bioluminescence after 3 hours of incubation, demonstrating a dose-dependent increase in bacterial activity with escalating levels of platelet depletion. Fig. 2-B *S. aureus* biomass after 24 hours of incubation, measured by absorbance at 585 nm on crystal violet assay.

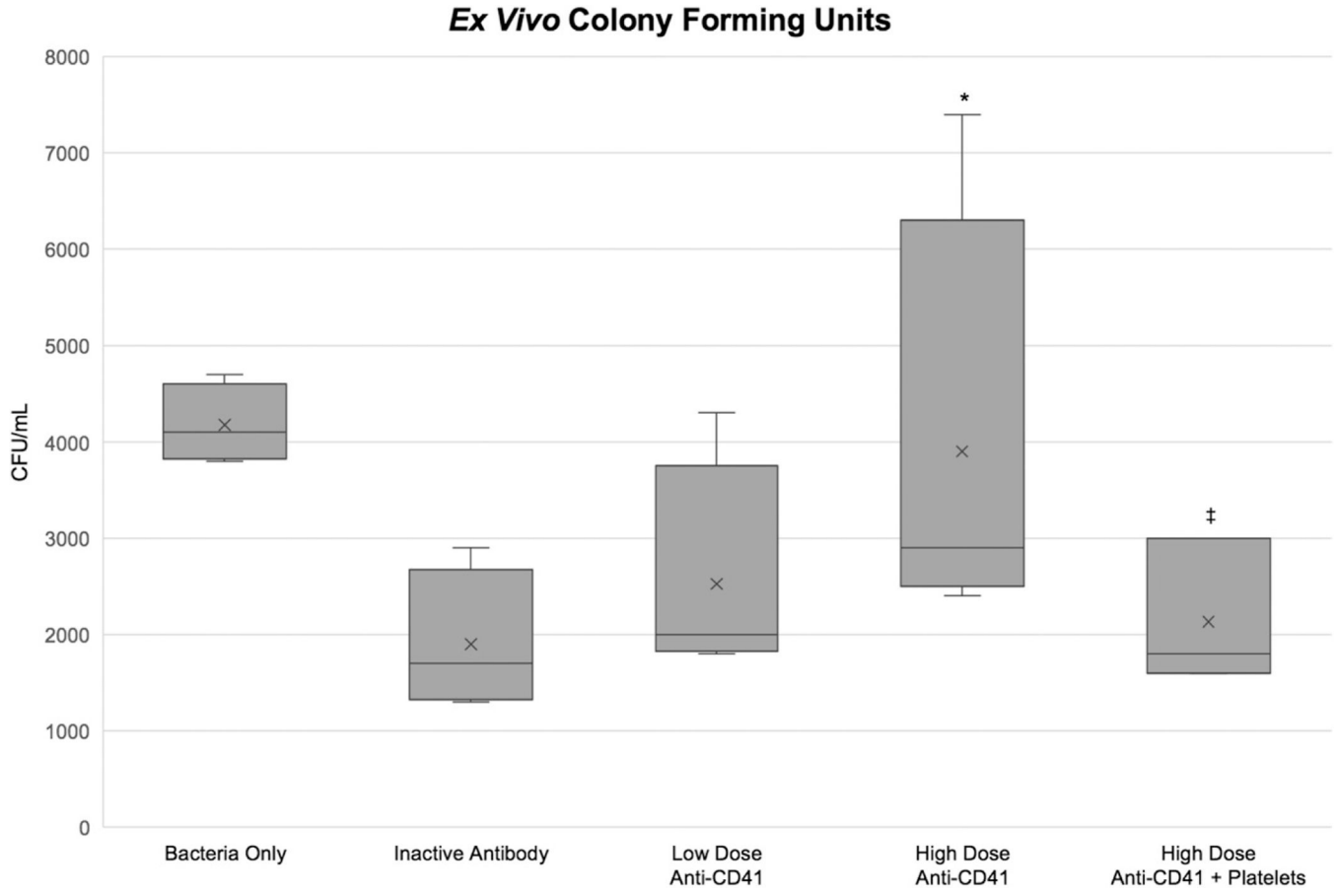

Fig. 3.

Box-and-whisker plot demonstrating ex vivo confirmation of bacterial burden via colony forming unit (CFU) quantification after overnight culture on TSB agar plates (n = 4 plates/group). The horizontal line within the boxes indicates the median, the outer borders indicate the interquartile range, while the whiskers indicate the minimum and maximum values of the data set. The mean value for each group is indicated by an ×. *P < 0.05 versus inactive antibody group. ‡P < 0.05 versus corresponding group without the addition of exogenous platelets.

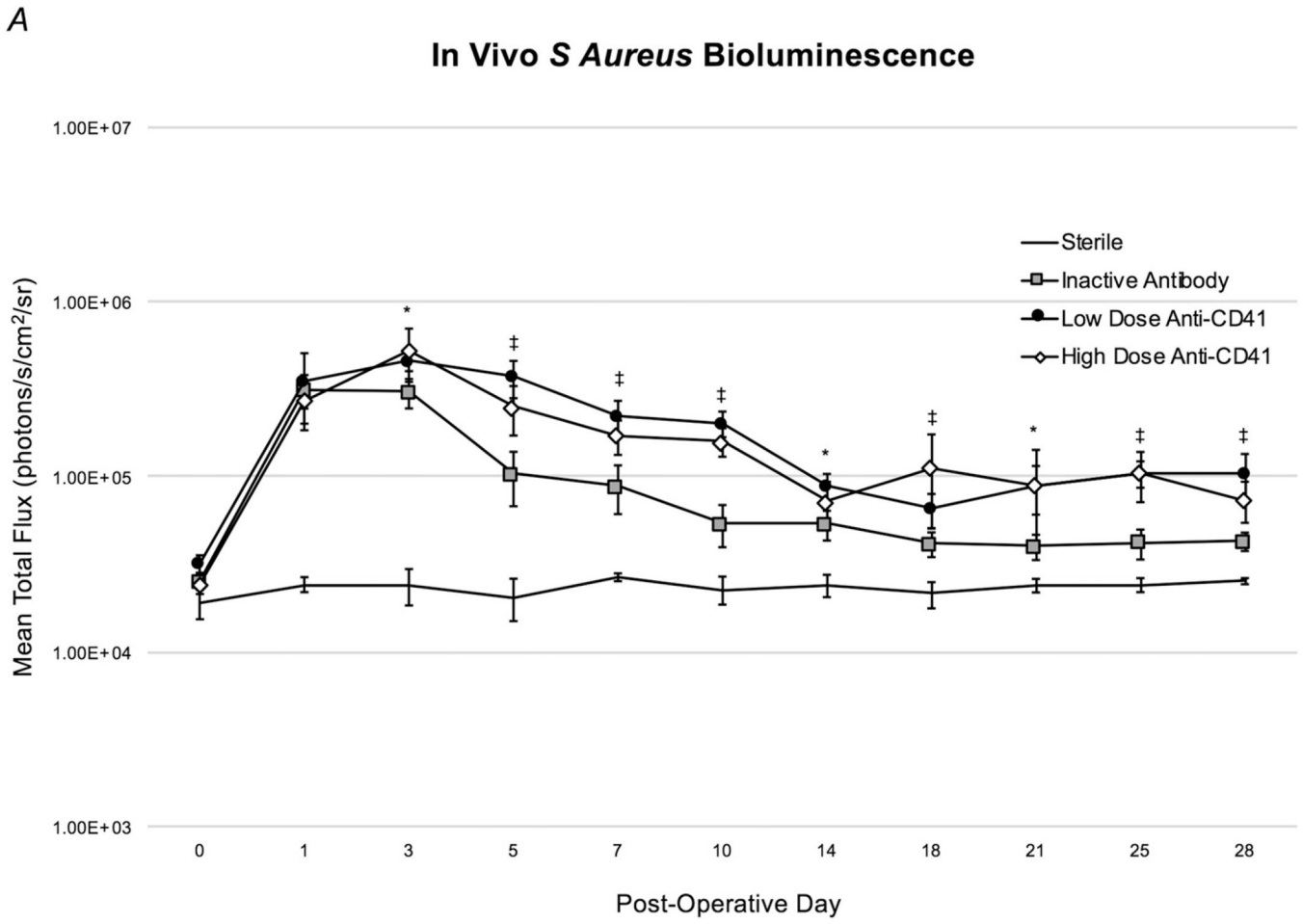

Fig. 4-A.

Figs. 4-A and 4-B Measurement of *S. aureus* bacterial burden in vivo using longitudinal live-animal bioluminescence imaging. Fig. 4-A Bacterial activity from POD0 until POD28, demonstrating significantly increased *S. aureus* burden in thrombocytopenic mice (IA group, n = 12 mice; LD and HD groups, n = 14 mice; sterile control group, n = 4 mice). Means and standard deviations are shown. *P < 0.05 for low-dose anti-CD41 only versus inactive antibody. ‡P < 0.05 for both low and high-dose anti-CD41 versus inactive antibody.

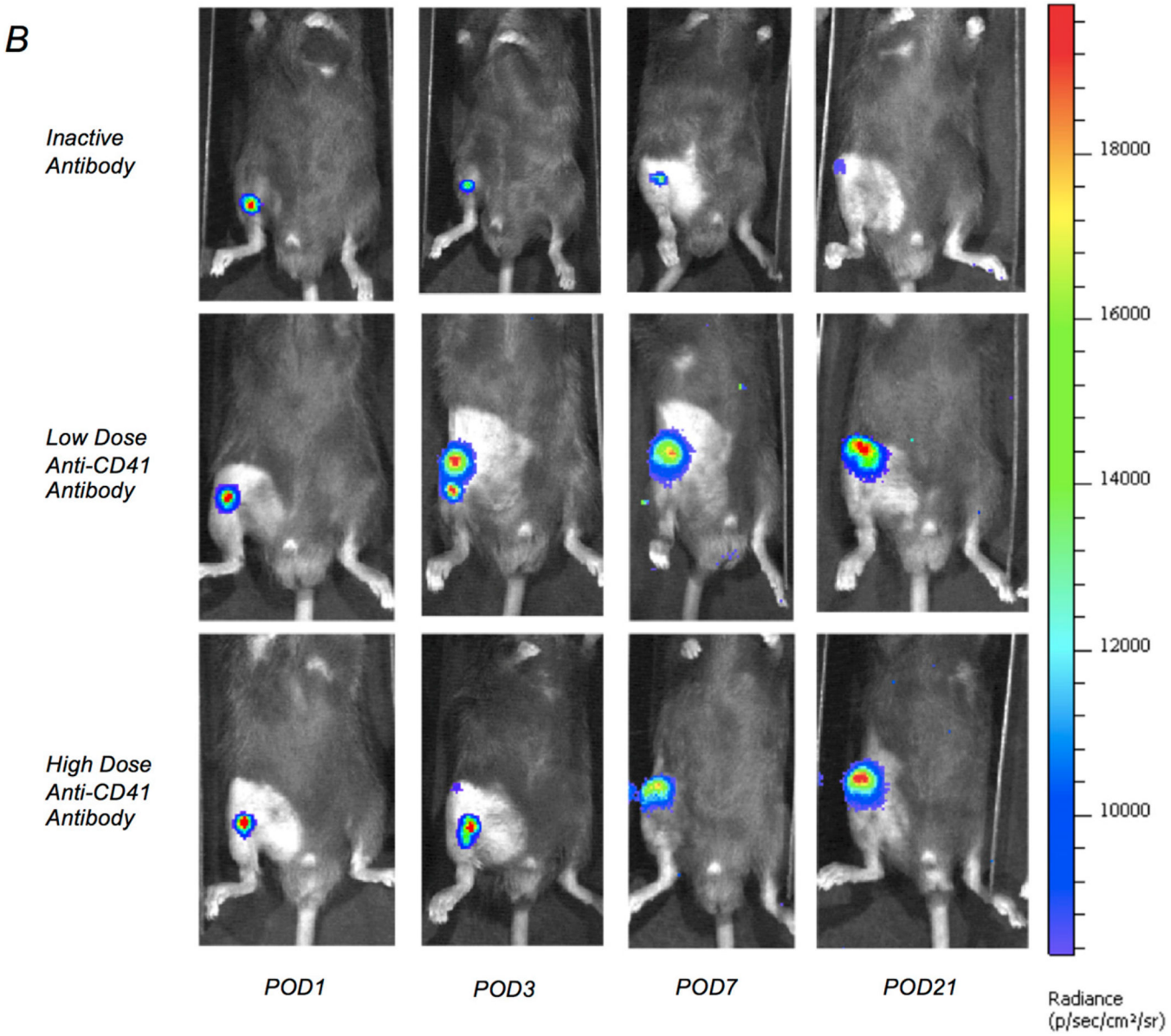

Fig. 4-B.

Fig. 4-B Representative in vivo *S. aureus* bioluminescent images at 3 selected postoperative time points.

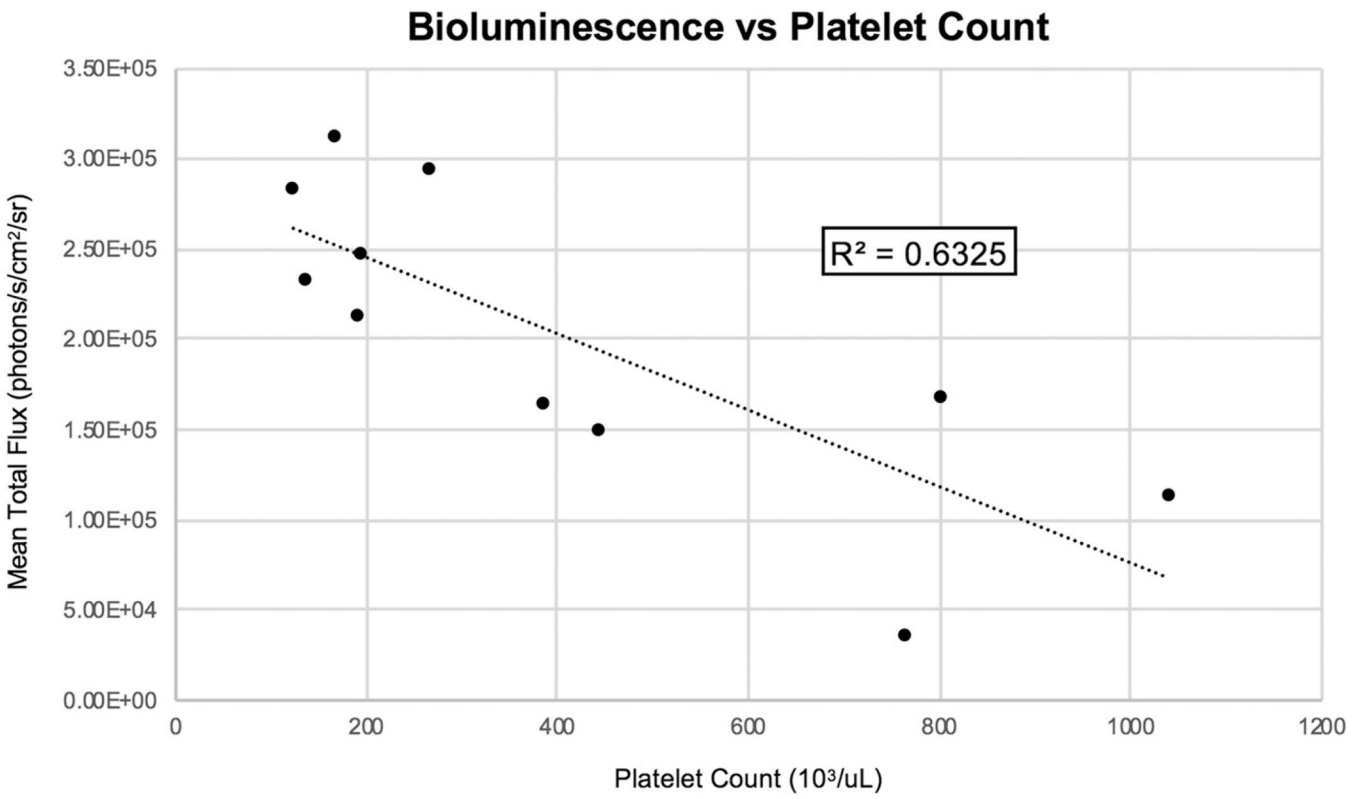

Fig. 5.

Platelet count versus bacterial burden. Platelet count at the time of surgery plotted against mean bacterial bioluminescence (mean total flux) over the entire study period (POD0 through POD28) revealed a significant negative correlation ($r^2 = 0.63$, $p = 0.037$) (n = 4 mice/group).

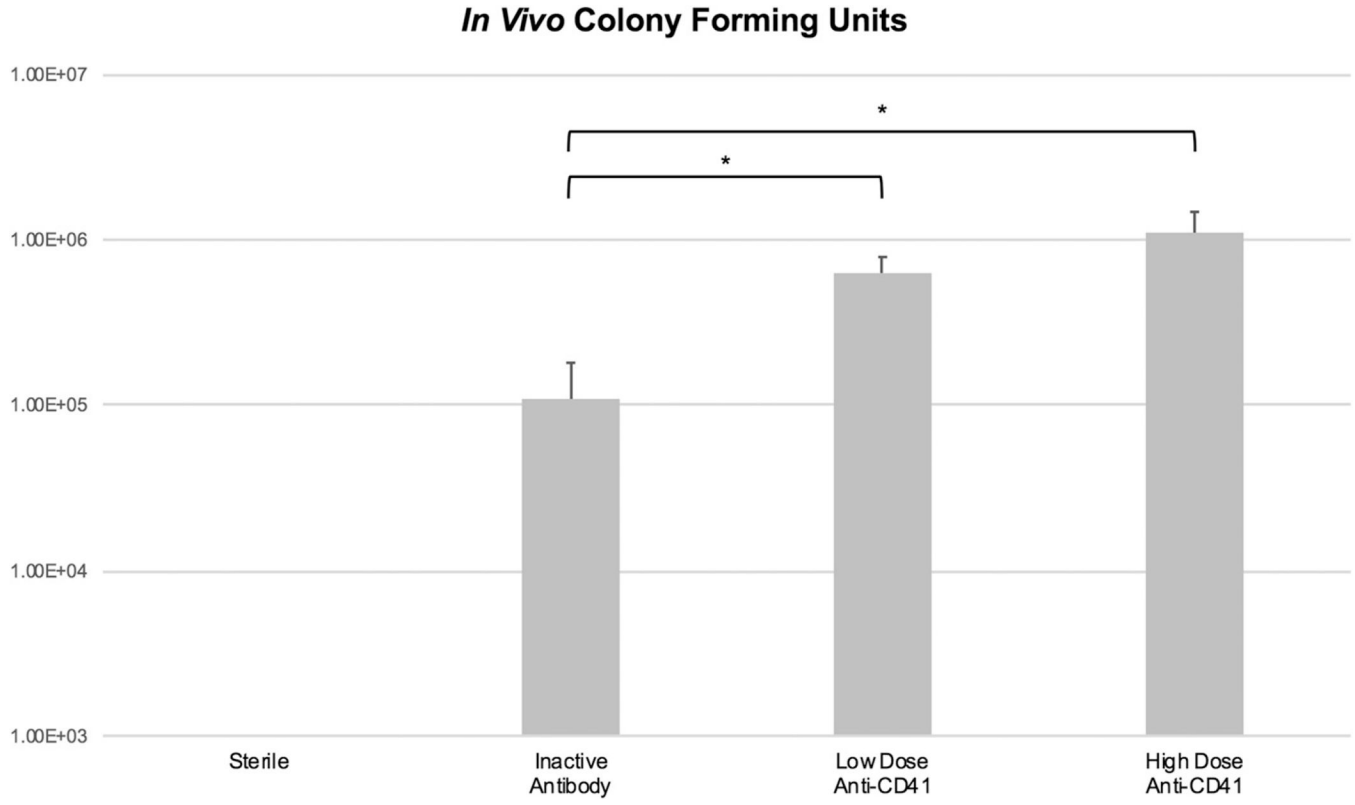

Fig. 6.

In vivo confirmation of bacterial burden using colony forming unit (CFU) quantification at postoperative day 28 (IA group, n = 12 mice; LD and HD groups, n = 14 mice; sterile control group, n = 4 mice). Mean CFU count of bacteria harvested from the surface of retrieved implants and from surrounding peri-implant tissue was significantly higher in thrombocytopenic mice (logarithmic scale). Means and standard deviations are shown. *P < 0.05 when comparing indicated groups.

TABLE I

Implant and Peri-Implant Tissue *S. aureus* Infection Rates on POD28 in Thrombocytopenic and Control Mice

	Implant Infection Rate*	P Value†	Tissue Infection Rate*	P Value†
Sterile	0.0% (0/4)	—	0.0% (0/4)	—
Inactive antibody	25.0% (3/12)	—	41.7% (5/12)	—
Low-dose anti-CD41	61.5% (8/13)	0.111	69.2% (9/13)	0.237
High-dose anti-CD41	72.7% (8/11)	0.039	72.7% (8/11)	0.214

*The number of implants or tissues that grew *S. aureus* out of the total number of mice in each group are depicted in parentheses.

†In comparison with inactive antibody group.