

ORIGINAL ARTICLE

Induced pluripotent stem cell-derived platelets kill multidrug-resistant *Staphylococcus aureus* via Toll-like receptor 2-MyD88 signaling and immunoglobulin G/FcγRIIA engagement

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Abstract

Background: Severe infection caused by multidrug-resistant bacteria, such as multidrug-resistant *Staphylococcus aureus* (MRSA), represents a pressing clinical challenge. While platelets are known to possess antibacterial activity against MRSA, the underlying mechanisms remain incompletely understood. In addition, the MRSA-killing capacity of induced pluripotent stem cell-derived platelets (iPSC-PLTs), which we succeeded in producing *ex vivo*, has not been previously characterized.

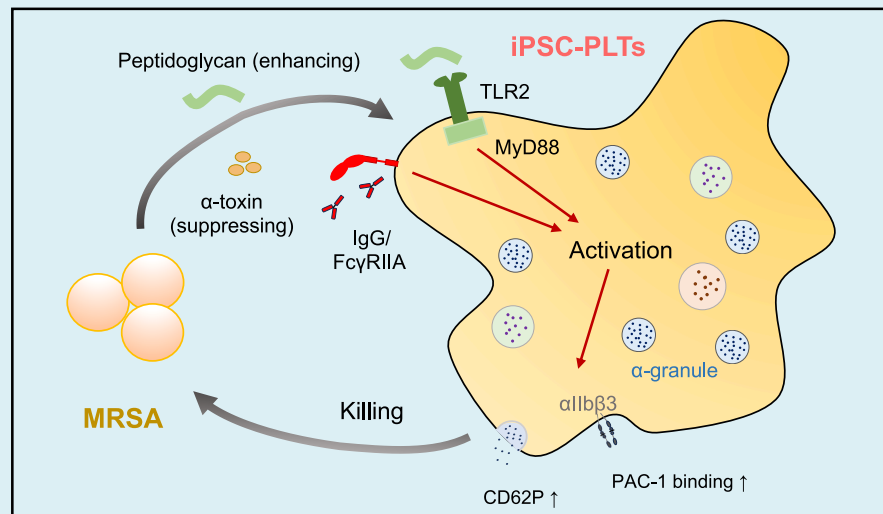
Objectives: We aimed to verify whether iPSC-PLTs are capable of killing MRSA and further elucidate the mechanisms involved in this process.

Methods: We performed *in vitro* colony assays to assess MRSA killing by iPSC-PLTs. To gain mechanistic insights, we applied antiplatelet agents, an FcγRIIA-blocking antibody, α-toxin-deficient MRSA, and MyD88-deficient iPSC-PLTs, which we created by gene editing.

Results: All 3 iPSC-PLT clones demonstrated MRSA-killing capacity. Although only minimal activation of iPSC-PLTs was observed, antiplatelet agents inhibited this killing. Notably, plasma components enhanced the bactericidal activity of iPSC-PLTs, in part via immunoglobulin G, as evidenced by inhibition with an FcγRIIA-blocking antibody. Compared with wild-type MRSA, α-toxin-deficient strains were more susceptible to iPSC-PLT-mediated killing, suggesting that α-toxin acts as a suppressor of this platelet function. Furthermore, MyD88-deficient iPSC-PLTs exhibited impaired MRSA-killing capacity, indicating the indispensable role of Toll-like receptor 2-mediated signaling in this response.

Conclusion: Collectively, our findings highlight the direct antimicrobial potential of iPSC-PLTs and provide mechanistic insights, particularly into the contribution of the Toll-like receptor-MyD88 axis. This study provided a basis for applying iPSC-PLTs as a novel therapeutic modality for combating MRSA infections and a genetically

modifiable platform for investigating unknown platelet function within the context of antimicrobial immunity.



Activation mechanism of induced pluripotent stem cell-derived platelets in antimultidrug-resistant *Staphylococcus aureus* responses.

Toll-like receptor (TLR) 2 ligand components of multidrug-resistant *Staphylococcus aureus* (MRSA), such as peptidoglycan, bind to TLR2 on platelets. The adaptor protein MyD88 elicits downstream signaling that induces platelet activation (as evidenced by increased PAC-1 binding and CD62P expression), leading to the killing of MRSA. Immunoglobulin (Ig) G/FcγR is also involved in activation, whereas MRSA α-toxin suppresses it, displaying the developmental competition between host defense and bacterial pathogenesis. iPSC-PLT, induced pluripotent stem cell-derived platelet

KEYWORDS

gene editing, MRSA, MyD88, platelets, Staphylococcal alpha-toxin, induced pluripotent stem cells, immunoglobulin G

Essentials

- The MRSA-killing capacity of iPSC-PLTs remains unaddressed.
- We performed *in vitro* MRSA-killing assays, applying inhibitors, ΔHla MRSA, and MyD88-deficient iPSC-PLTs.
- iPSC-PLTs killed MRSA involving activation, IgG-FcγR and TLR-MyD88 axis, but suppressed by Hla.
- iPSC-PLTs are applicable for MRSA infections and gene ediatable for investigation purposes.

1 | INTRODUCTION

Staphylococcus aureus colonizes multiple human body sites and is a leading cause of community- and hospital-acquired infections, including life-threatening infections such as endocarditis and central line-associated bloodstream infection [1]. Morbidity, mortality, length of stay, and associated health care costs are further exacerbated by the emergence and ongoing spread of multidrug-resistant *S aureus* (MRSA) [1]. Platelets—small anucleate cells essential for hemostasis and thrombosis—have also been implicated in host defense against infection,

and recent reports describe their anti-MRSA activity, although the underlying mechanisms remain incompletely defined [2,3].

We previously achieved *ex vivo* clinical-scale production of induced pluripotent stem cell-derived platelets (iPSC-PLTs) using an immortalized megakaryocyte progenitor cell line (imMKCL) system. imMKCLs are established by introducing doxycycline (Dox)-inducible c-MYC, BMI1, and BCL-XL transgenes into hematopoietic/megakaryocytic progenitors differentiated from human iPSCs [4]. Under culture condition with addition of Dox, imMKCLs proliferate; upon Dox withdrawal (Dox-OFF), they mature and release iPSC-PLTs.

Development of a turbulent-flow bioreactor and adjunctive small molecules enabled large-scale *ex vivo* production ($>10^{11}$ iPSC-PLTs), culminating in the first-in-human clinical trial of iPSC-PLTs [5–7]. The platform also supports additional modifications via gene editing, such as β 1-tubulin-Venus reporter overexpression and β 2-microglobulin knockout (KO) [8,9]. Because iPSC-PLTs can be produced in a uniform, off-the-shelf manner and endowed with specific functions through gene editing and other modalities, they represent a tractable cell product for previously unexplored applications, including anti-MRSA therapy. Whether iPSC-PLTs possess anti-MRSA activity comparable to that of endogenous platelets, however, has not been systematically assessed.

In this study, we demonstrate that iPSC-PLTs directly kill MRSA in an *in vitro* coculture assay. We then delineate the contributing mechanisms by leveraging iPSC-PLTs with MyD88-KO, given MyD88's critical role as an adaptor for innate immune sensing [10]. Under plasma-free conditions, MRSA induced low-grade iPSC-PLT activation, yet killing still occurred and was suppressed by anti-platelet agents. Human plasma enhanced iPSC-PLT-mediated MRSA killing, in part via immunoglobulin (Ig) G engagement of Fc γ RIIA, as shown by blockade with an Fc γ RIIA-specific antibody. Compared with wild-type (WT) MRSA, an α -toxin-deficient (Δ Hla) mutant was more susceptible to killing, indicating that α -toxin acts as a suppressor—not a stimulant—of iPSC-PLT antimicrobial activity. Finally, MyD88-KO iPSC-PLTs showed diminished MRSA killing and reduced activation vs WT iPSC-PLTs, implicating Toll-like receptor (TLR) 2-MyD88 signaling in platelet sensing and effector function. Collectively, these findings revealed the direct anti-MRSA potential of iPSC-PLTs and provided a mechanistic basis for their further development as an adjunctive therapy.

2 | METHODS

2.1 | Ethics

The use of human iPSCs, imMKCLs, and peripheral blood from healthy volunteers was approved by the Kyoto University ethics committees. Human platelets and plasma were used in compliance with the Ministry of Health, Labor and Welfare of Japan's "Guidelines on the Use of Donated Blood in R&D."

2.2 | Human plasma

Human plasma provided by the Japanese Red Cross Society was pooled, clarified by centrifugation (2100 rpm for 20 minutes at 1 °C) to remove cryoglobulin, stored at -30 °C, and, prior to experiments, sequentially filtered (0.45 μ m filter, Millipore, #SLHVR33RB, and 0.22 μ m filter, Millipore, #SLGPR33RB) to remove impurities.

2.3 | Human peripheral blood platelets

Human peripheral blood platelets (PB-PLTs) were obtained from the Japanese Red Cross Society or prepared from the peripheral blood of healthy donors after centrifugation. Platelets were then isolated by centrifuging at 1500 rpm for 15 minutes with low brake. Precipitated platelets were collected, resuspended in plain RPMI-1640 medium (Nacalai Tesque, #05176-25) at room temperature, and used within an hour.

2.4 | imMKCLs and iPSC-PLTs

imMKCLs (SeV2 clone 7, NIH-5, and M35-1) were generated from human iPSCs and cultured as described [4,5,7]. Dox-ON supported proliferation, and Dox-OFF induced maturation and platelet release.

2.5 | MyD88-KO

Following a previously published re-reprogramming method [8,9], MyD88 was disrupted in iPSCs re-reprogrammed from SeV2 imMKCLs using clustered regularly interspaced short palindromic repeats/CRIPR associated protein (CRISPR/Cas)9 technology (GenAhead Bio Inc). In order to obtain iPSC-PLTs depleted of the MyD88 gene, iPSCs re-reprogrammed from imMKCL SeV2 were transfected with the following plasmids using a NEPA21 electroporation system (Nepagene): a plasmid with DNA sequences of single guide RNA (sgRNA) targeting the MyD88 gene and another with Cas9 derived from *Streptococcus pyogenes* (SpCas9) for MyD88 gene depletion; and also a plasmid encoding puromycin-N-acetyltransferase to endow puromycin resistance in successfully transduced cells. Puromycin (0.6 or 1.0 μ g/mL) was added the following day. After T7E1 mismatch assay screening, single-cell cloning, and genotypic polymerase chain reaction, 3 clones (exon 3-targeted: numbers 28, 30, and 37) were homozygous MyD88-KO, and 1 clone (exon 1-targeted: number 4) did not knock out the gene.

sgRNA and primers for the T7E1 assay and genotyping were as follows:

sgRNA for exon 1: 5'-CTTGAACGTGCGGACACAGG -3';
 sgRNA for exon 3: 5'-ATGAAGGCATCGAAACGCTC-3';
 primer exon 1-F: 5'-CGTTTCCTACAACCCCGAA-3';
 primer exon 1-R: 5'-TTCCTTCCCATCTCCGCTA-3';
 primer exon 3-F: 5'-ACTTCTCAGAGCCGTTGAGC-3';
 primer exon 3-R: 5'-CGTTTCCTACAACCCCGAA-3'.

2.6 | Bacterial strains and culture

MRSA USA300 (TCH1516) and an isogenic Δ Hla mutant were used [3]. Strains were grown in Todd-Hewitt broth (THB; Sigma-Aldrich,

#T1438) at 37 °C with shaking to mid-log phase (optical density [OD]₆₀₀ ≈ 0.4), pelleted (8000 rpm for 10 minutes), washed twice in phosphate-buffered saline, pelleted again (12,000 rpm for 7 minutes), and finally resuspended in RPMI-1640 media.

2.7 | MRSA-killing assay

iPSC-PLTs or PB-PLTs (1×10^7) were coincubated with 10^5 colony-forming units (CFUs) of MRSA in 200 µL of RPMI-1640 medium for 2 hours at 37 °C. Suspensions were triturated with a pipette, serially diluted, and plated on THB agar; plates were incubated overnight at 37 °C. Surviving CFUs were enumerated, and percent killing was calculated relative to the input inoculum. A high-speed micro-centrifuge (MX-307, TOMY Lab Equipment) was used as needed to separate bacteria. At least 3 independent experiments were performed, each with at least 3 biological replicates for PB-PLTs.

2.8 | Platelet pretreatments and stimulation

Before the killing assays, 1×10^7 platelets were pretreated for 30 to 60 minutes at 37 °C with ticagrelor (Cayman Chemical, #15425), BPTU (MedChemExpress, #HY-13831), aspirin (Sigma-Aldrich, #A5376), tirofiban (MedChemExpress, #HY-17369), or anti-FcγRIIA monoclonal antibody IV.3 (STEMCELL Technologies, #60012) at the indicated concentrations. The TLR2 ligands used for stimulation were peptidoglycan (PGN; Sigma-Aldrich, #77140) and Pam3CSK4 (InvivoGen, #tlrl-pms).

2.9 | Bacterial proliferation curves

Growth kinetics were assessed for the WT MRSA USA300 strain TCH1516 and its isogenic ΔHla mutant. Overnight cultures were diluted into THB at 37 °C with shaking to a starting OD₆₀₀ of 0.1. Cultures were incubated at 37 °C with shaking, and OD₆₀₀ was measured every 30 minutes for 6 hours using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific).

2.10 | Flow cytometric analysis

The following fluorophore-conjugates and antibodies were used. From BD Biosciences: FITC antihuman PAC-1 (#340507), FITC annexin V (#556419), and BV421 antihuman CD107A (clone H4A3, #566261); from BioLegend: FITC antihuman CD282 (TLR2, clone W15145C, #392307), PE antihuman CD284 (TLR4, clone HTA125, #312805), APC antihuman CD62P (P-selectin, clone AK4, #304910), FITC mouse IgM, κ isotype control (clone MM-30, #401606), APC mouse IgG1, κ isotype control (clone MOPC-21, #400120), PE antihuman CD41 (clone HIP8, #303706), and APC antihuman CD41 (clone HIP8, #303710); and from Invitrogen: PE antihuman CD289

(TLR9, clone eB72-1665, #12-9099-82) and PE-Cyanine7 antihuman CD63 (clone H5C6, #25-0639-42). For staining, platelets were diluted 50-fold in Tyrode-HEPES buffer and incubated at room temperature for 20 minutes. For annexin V binding assays, cells were diluted 50-fold in annexin V binding buffer and incubated under the same conditions as the staining. Data were acquired on a FACSLytic flow cytometer (BD Biosciences) and analyzed using FlowJo 10.8.1 (BD Biosciences).

2.11 | Calcium assay (Fluo-4 assay)

Intracellular calcium (Ca^{2+}) mobilization was measured using Fluo-4 AM (BD Pharmingen, #565878). Washed platelets (iPSC-PLTs or PB-PLTs) were resuspended in RPMI-1640 medium at 1×10^7 /mL and incubated with 5 µM Fluo-4 AM for 20 minutes at room temperature. Fluorescence intensity was recorded before and after stimulation with PGN or Pam3CSK4 at the indicated concentrations, with 1 mM ethylene glycol tetraacetic acid as a negative control. Time-course fluorescence changes were collected on a BD FACSLytic cytometer. Each condition was measured in at least 3 independent experiments, with triplicate samples per experiment.

2.12 | ELISA and chemiluminescent enzyme immunoassay

After a 2-hour coincubation of platelets with MRSA (USA300 WT), supernatants were collected by centrifugation at 1000 rpm for 5 minutes. Platelet factor 4 (PF4) and β-thromboglobulin (β-TG) were quantified using commercial ELISA kits (R&D Systems, #DY795 and PeproTech, #900-K40) according to the manufacturer's instructions, with samples diluted 1:50 in phosphate-buffered saline. CLEC-2 shedding was assessed by a chemiluminescent enzyme immunoassay using the STACIA system (LSI Medience), as previously described [11]. Briefly, magnetic particles were coated with the anti-CLEC-2 monoclonal antibody 11D5. Supernatant samples were then incubated with antibody-coated magnetic particles. After washing, the particles were further incubated with an alkaline phosphatase-conjugated anti-CLEC-2 monoclonal antibody 11E6. After washing again, the magnetic particles were incubated with a chemiluminescent substrate solution (CDP-Star, Applied Biosystems, Thermo Fisher Scientific), and luminescence was measured using a luminometer installed in the STACIA system.

2.13 | Protein sample preparation

Cells were centrifuged to remove culture medium, then homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4; Fujifilm, #316-90221), 150 mM NaCl (Fujifilm, #191-01665), 0.5% Triton X-100 (Sigma-Aldrich, #X100), 2 mM EDTA (pH 8.0; Nacalai Tesque, #06894), and protease inhibitor cocktail (Roche, #5892970001) for

30 minutes on ice. Then, the cell lysate was centrifuged for 15 minutes at 12,000 rpm at 4 °C to obtain the supernatant.

2.14 | Western blot analysis

Protein samples were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, #23225), and Western blotting was performed using the ProteinSimple Reagent Kit (SM-W004, ProteinSimple) and Wes equipment (ProteinSimple) for fully automated measurements and analysis. The following antibodies were used: anti- β -actin mouse monoclonal antibody (Sigma-Aldrich, #A1978) and anti-MyD88 rabbit polyclonal antibody (Cell Signaling Technology, #3699).

2.15 | Statistical analysis

Continuous variables are presented as mean $\pm$ SEM. Data were tested for normality and equal variance prior to parametric analysis. Comparisons between 2 groups were performed using unpaired Student's *t*-tests (2-tailed) in Prism 8 software (GraphPad). A *P* value $< .05$ was considered statistically significant.

3 | RESULTS

3.1 | iPSC-PLTs possess MRSA-killing activity with low-grade activation

To evaluate whether iPSC-PLTs display antibacterial function, we performed an *in vitro* MRSA-killing assay in which platelets were cocubated with *S aureus* USA300 for 2 hours, followed by CFU enumeration on agar plates (Figure 1A). Three independent iPSC-PLT clones (Sev2, NIH5, and M35-1) exhibited MRSA killing comparable with that of PB-PLTs (Figure 1B). Additionally, Sev2 iPSC-PLTs displayed significant killing activity against 2 other MRSA strains (MR0001 and MR0002), but not against a methicillin-susceptible *Staphylococcus aureus* strain (Supplementary Figure 1).

We next examined whether iPSC-PLTs undergo conventional activation in response to MRSA coculture. Flow cytometry was used to measure the established platelet activation markers CD62P (α -granule release), CD63 (dense granule release), CD107a (lysosomal degranulation), PAC-1 binding (activation of $\alpha_{IIb}\beta_3$ integrin), and annexin V binding (phosphatidylserine exposure). Positive controls with adenosine diphosphate/thrombin receptor activator peptide 6 confirmed assay responsiveness, with robust induction of all markers and the release of PF4 and β -TG (Supplementary Figure 2). MRSA cocubation induced more modest changes: PAC-1 binding was significantly increased in Sev2 and M35-1 iPSC-PLTs, and PF4/ β -TG release reached significance in some conditions but remained low overall (Figure 1C, D, and Supplementary Figure 3).

CLEC-2 shedding, another platelet activation readout [11], showed a slight but significant increase in Sev2 iPSC-PLTs (Figure 1E).

Importantly, treatment with platelet inhibitors (tirofiban, aspirin, BPTU, and ticagrelor) significantly reduced MRSA killing (Figure 1F), suggesting that low-level platelet activation functionally contributes to antibacterial activity.

3.2 | Human plasma enhances MRSA-killing activity by iPSC-PLTs partly via IgG

Given that platelets function in plasma-rich environments, we next tested the impact of human plasma on MRSA growth and platelet killing. The addition of 2.5% plasma alone enhanced MRSA proliferation, consistent with nutrient supplementation, whereas the inclusion of plasma in coculture with Sev2 iPSC-PLTs significantly increased bacterial killing (Figure 2A). The same plasma addition seemingly had little effect on PB-PLTs (Supplementary Figure 3B).

Flow cytometry demonstrated that plasma alone (without MRSA) increased CD62P expression and PAC-1 binding in iPSC-PLTs (Figure 2B) but reduced annexin V binding in both iPSC-PLTs and PB-PLTs (Figure 2B and Supplementary Figure 3C). These findings suggest that plasma factors enhance platelet viability and hemostasis-associated activation, thereby augmenting antimicrobial activity.

Proteomic profiling further revealed that PB-PLTs with high MRSA-killing activity were enriched for signatures of an "Ig-mediated immune response" (Supplementary Figure 4). Based on this observation, we tested whether Fc γ RIIA, the platelet receptor for IgG, was required. Pretreatment with the Fc γ RIIA-blocking antibody IV.3 significantly reduced MRSA killing by iPSC-PLTs, both in the presence and absence of plasma (Figure 2C). Thus, IgG interactions via Fc γ RIIA enhance platelet-mediated killing of MRSA.

3.3 | α -Toxins suppress MRSA killing by iPSC-PLTs

We next examined whether α -toxin, a pore-forming virulence factor secreted by *S aureus*, modulates platelet antibacterial activity [3]. Using an isogenic Δ Hla mutant of USA300, we observed slightly faster bacterial growth compared with WT MRSA (Figure 3A). Nevertheless, Δ Hla MRSA was more susceptible to killing by iPSC-PLTs, with a CFU recovery consistently lower than WT MRSA (63.07 ± 5.48 vs 67.20 ± 11.24 ; Figure 3B). Activation markers (CD62P, PAC-1, and annexin V) were similar between WT and Δ Hla MRSA (Figure 3C). These results suggest that α -toxin dampens platelet antimicrobial activity without a global effect on platelet activation status.

3.4 | The TLR2-MyD88 pathway is involved in MRSA-killing activity by iPSC-PLTs

Because platelets express innate immune receptors, we investigated whether the platelet response to MRSA is mediated by TLR signaling

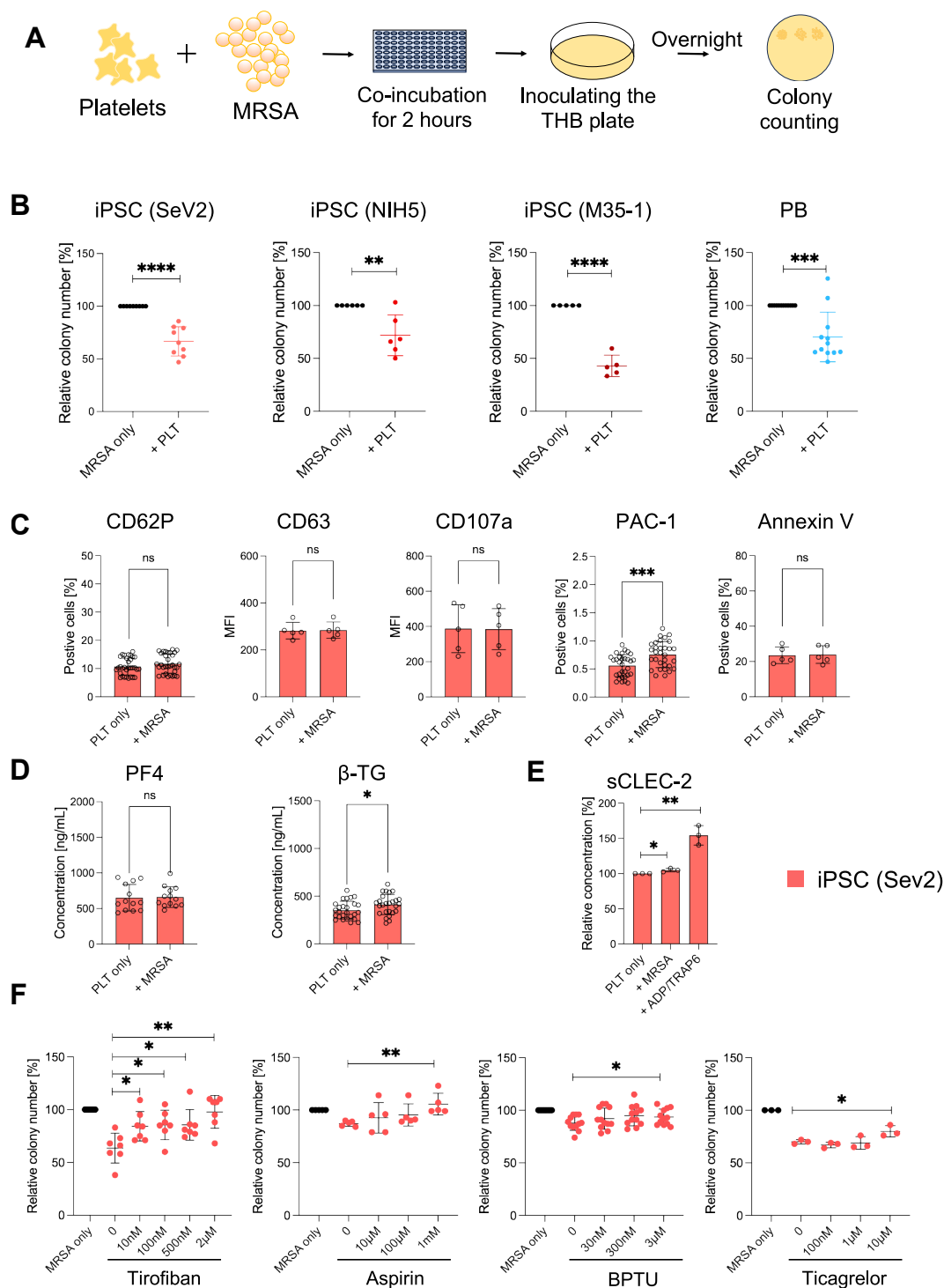


FIGURE 1 Induced pluripotent stem cell (iPSC)-derived platelets (iPSC-PLTs) exhibit antimultidrug-resistant *Staphylococcus aureus* (MRSA) activity. (A) Schema of the MRSA-killing assay. Colony formation units on Todd-Hewitt broth (THB) agar plates were counted. (B) MRSA-killing assay using 3 iPSC-PLT clones (Sev2, NIH5, and M35-1) and healthy peripheral blood (PB) platelets (PLTs). MRSA killing was evaluated by quantifying MRSA colony numbers relative to the MRSA-only control as a percentage. (C–E) SeV2 iPSC-PLTs with or without 2-hour coincubation with MRSA were subject to flow cytometry analysis for the (C) expression of CD62P, CD63, and CD107a and the binding of PAC-1 and annexin V, (D) enzyme-linked immunosorbent assay for the release of platelet factor 4 (PF4) and β-thromboglobulin (β-TG), and (E) chemiluminescent enzyme immunoassay for the release of sCLEC-2. (F) MRSA-killing assay using SeV2 iPSC-PLTs pretreated with gradient concentrations of tirofiban, aspirin, BPTU, or ticagrelor. Compiled data of 3 independent experiments. Data are presented as means ± SEM. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, ADP, adenosine diphosphate; ns, not significant. MFI, mean fluorescence intensity; sCLEC-2, soluble C-type lectin-like receptor 2; TRAP-6, thrombin receptor activator peptide 6.

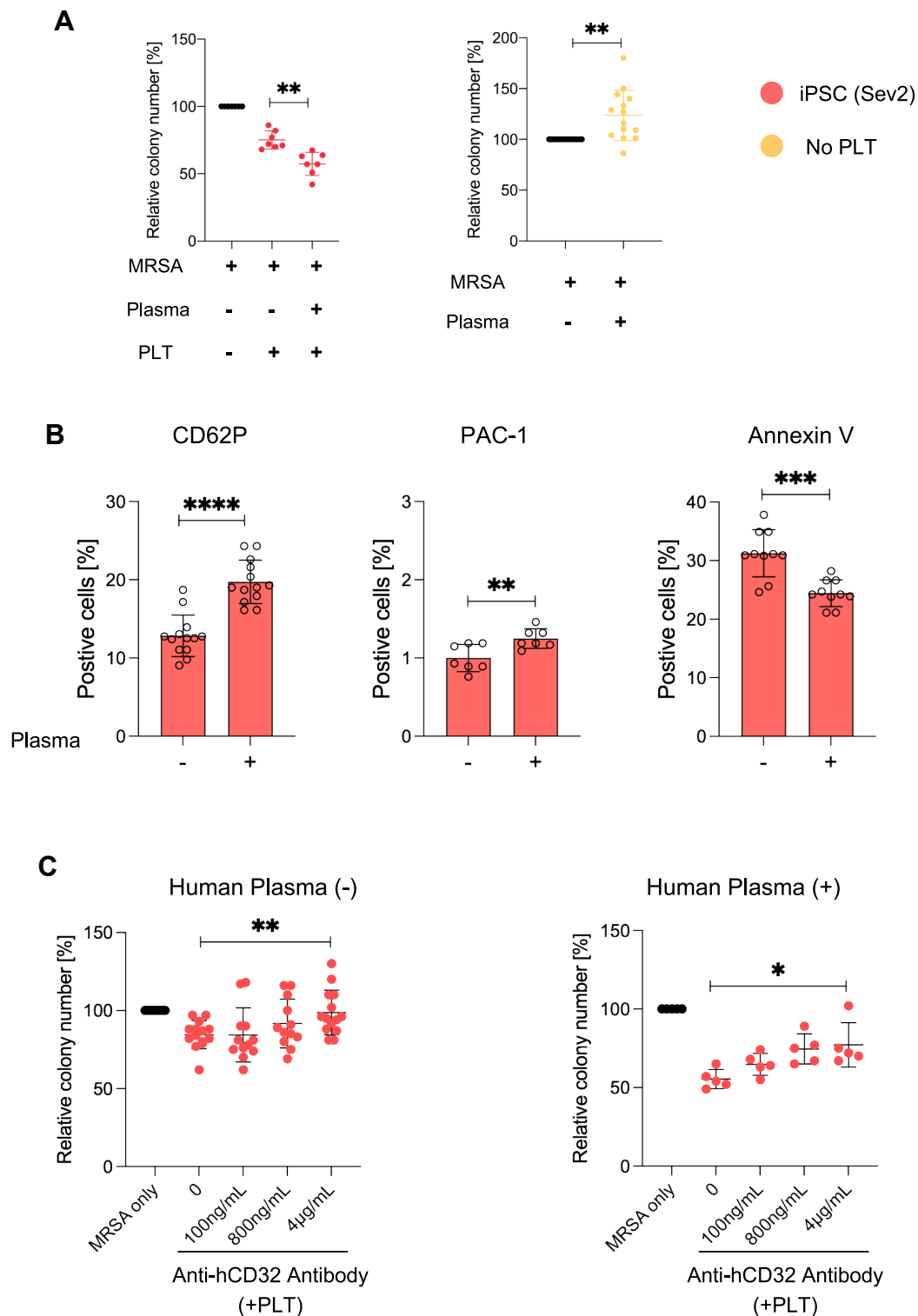


FIGURE 2 Human plasma enhances multidrug-resistant *Staphylococcus aureus* (MRSA) killing by induced pluripotent stem cell (iPSC)-derived platelets (iPSC-PLTs) via immunoglobulin G. (A) MRSA-killing assay with or without the addition of human plasma for iPSC-PLTs (Sev2) or no platelet (PLT; control) condition. (B) Flow cytometry analysis of CD62P expression, PAC-1 binding, and annexin V binding in Sev2 treated with or without human plasma. (C) MRSA-killing assay using Sev2 pretreated with antihuman CD32 (FcγRIIA) antibody in the absence or presence of human plasma. Compiled data of 5 independent experiments. Data are presented as means $\pm$ SEM. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

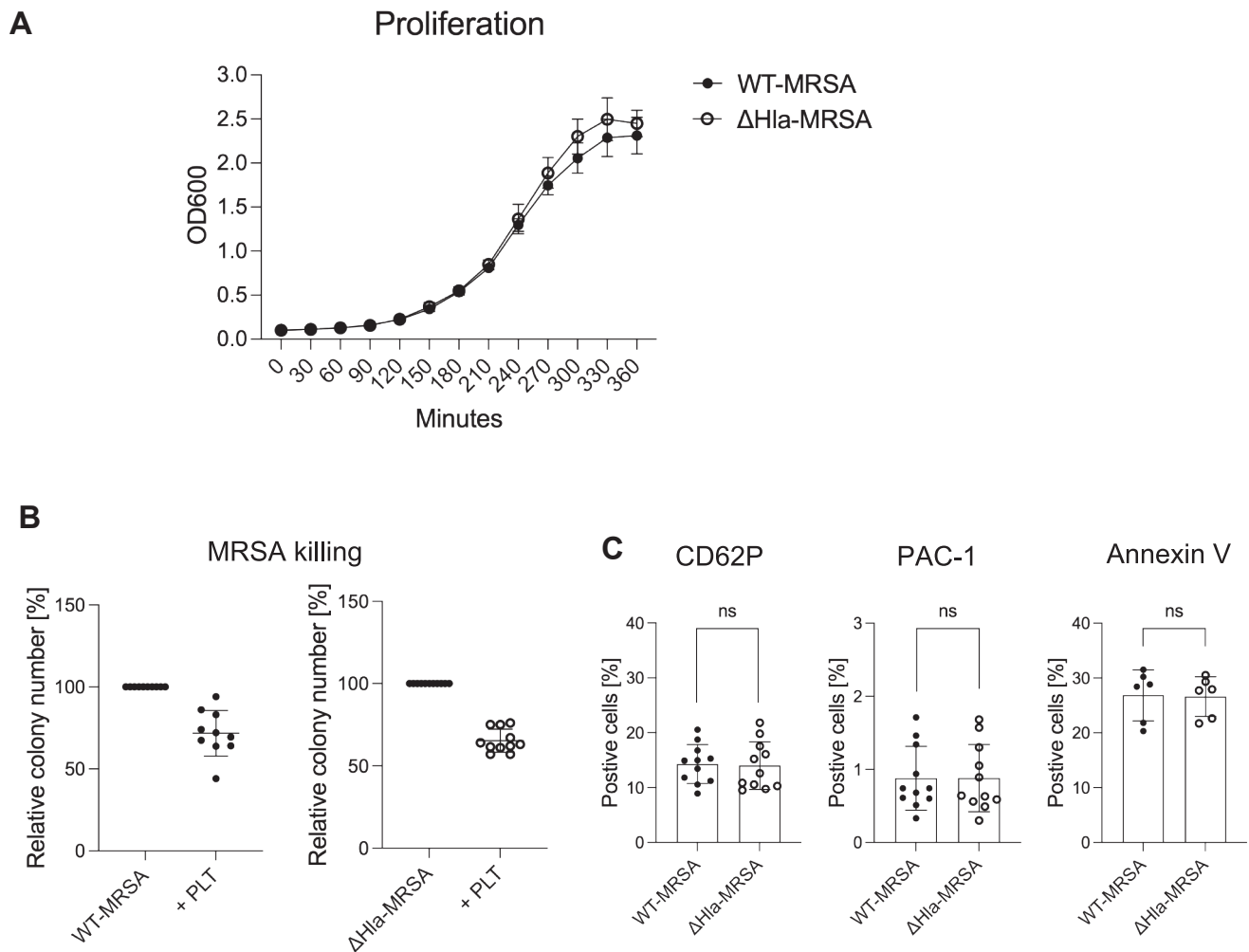


FIGURE 3 Multidrug-resistant *Staphylococcus aureus* (MRSA) α -toxin suppresses the killing of MRSA by induced pluripotent stem cell-derived platelets (iPSC-PLTs). (A) Growth curves of wild-type (WT) and α -toxin-deficient (Δ Hla)-MRSA measured by changes in the absorbance of optical density (OD)₆₀₀. (B) MRSA-killing assay of iPSC-PLTs. (C) Flow cytometry analysis of CD62P expression, PAC-1 binding, and annexin V binding in iPSC-PLTs coincubated with WT MRSA or Δ Hla-MRSA. Compiled data of 3 independent experiments. Data are presented as means $\pm$ SEM. ns, not significant; PLT, platelet.

[12–15]. Flow cytometry confirmed that iPSC-PLTs express TLR2 and TLR4, similar to PB-PLTs (Figure 4A and Supplementary Figure 5A). Given that cell wall components of Gram-positive bacteria are the major pathogen-associated molecular patterns recognized through TLR2 [10], we treated iPSC-PLTs and PB-PLTs with the TLR2 ligands peptidoglycan (PGN) and Pam3CSK4. High concentrations of PGN and Pam3CSK4 induced robust activation, as shown by increased CD62P, PAC-1, and annexin V staining (Figure 4B–D and Supplementary Figure 5B–D), and triggered intracellular Ca^{2+} mobilization (Figure 4E, F, and Supplementary Figure 5E, F).

To directly test the role of TLR2 and its downstream signaling, we established iPSC-PLTs deficient in MyD88, an adaptor protein that exclusively conveys downstream signaling of most TLRs, including TLR2 [10]. Due to the difficulty in the genetic manipulation of imMKCLs, we performed MyD88-KO by a re-reprogramming

procedure we previously adopted [9]. The MyD88 gene was knocked out in iPSCs re-reprogrammed from imMKCLs using CRISPR/Cas9, and then re-differentiated to imMKCLs to obtain MyD88-KO imMKCL clones (Figure 5A). We confirmed that MyD88 was successfully knocked out in clones 28, 30, and 37, which targeted exon 3, but not in clone 4, which targeted exon 1 of MyD88 (Figure 5B).

MRSA killing by MyD88-KO iPSC-PLTs was significantly diminished compared with WT iPSC-PLTs (Figure 5C). Activation levels of MyD88-KO iPSC-PLTs, as measured by CD62P and PAC-1 binding, were also lower than those of WT iPSC-PLTs. No intergroup differences were observed in annexin V binding levels (Figure 5C). These results indicate that TLR2-MyD88 signaling contributes to platelet activation and MRSA killing, although additional sensing pathways are likely involved.

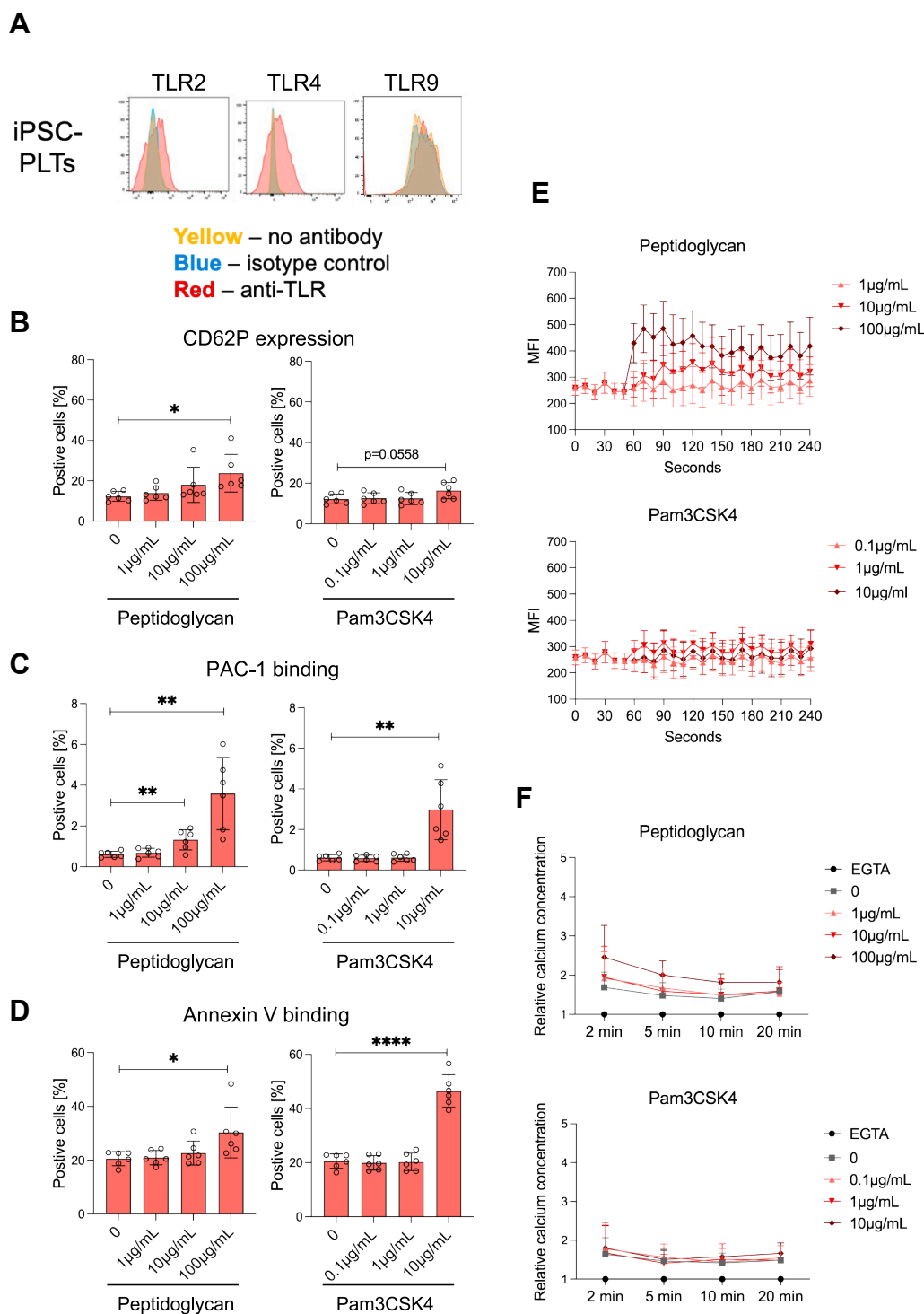


FIGURE 4 Induced pluripotent stem cell-derived platelets (iPSC-PLTs; Sev2) are activated by Toll-like receptor (TLR) 2 agonists. (A) Flow cytometry analysis of TLR2, TLR4, and TLR9 expression on Sev2. (B–D) Flow cytometry analysis of CD62P expression, PAC-1 binding, and annexin V binding in Sev2 following stimulation with increasing concentrations of Pam3CSK4 or peptidoglycan. (E) Flow cytometry analysis of intracellular calcium levels in Sev2 following stimulation with a TLR2 agonist (Pam3CSK4 or peptidoglycan). (F) Long-term measurements of intracellular calcium concentration in Sev2 stimulated with a TLR2 agonist (Pam3CSK4 or peptidoglycan). Values are relative to levels in the presence of ethylene glycol tetraacetic acid (EGTA). Compiled data of 3 independent experiments. Data are presented as means $\pm$ SEM. * $P < .05$, ** $P < .01$, **** $P < .0001$. MFI, mean fluorescence intensity.

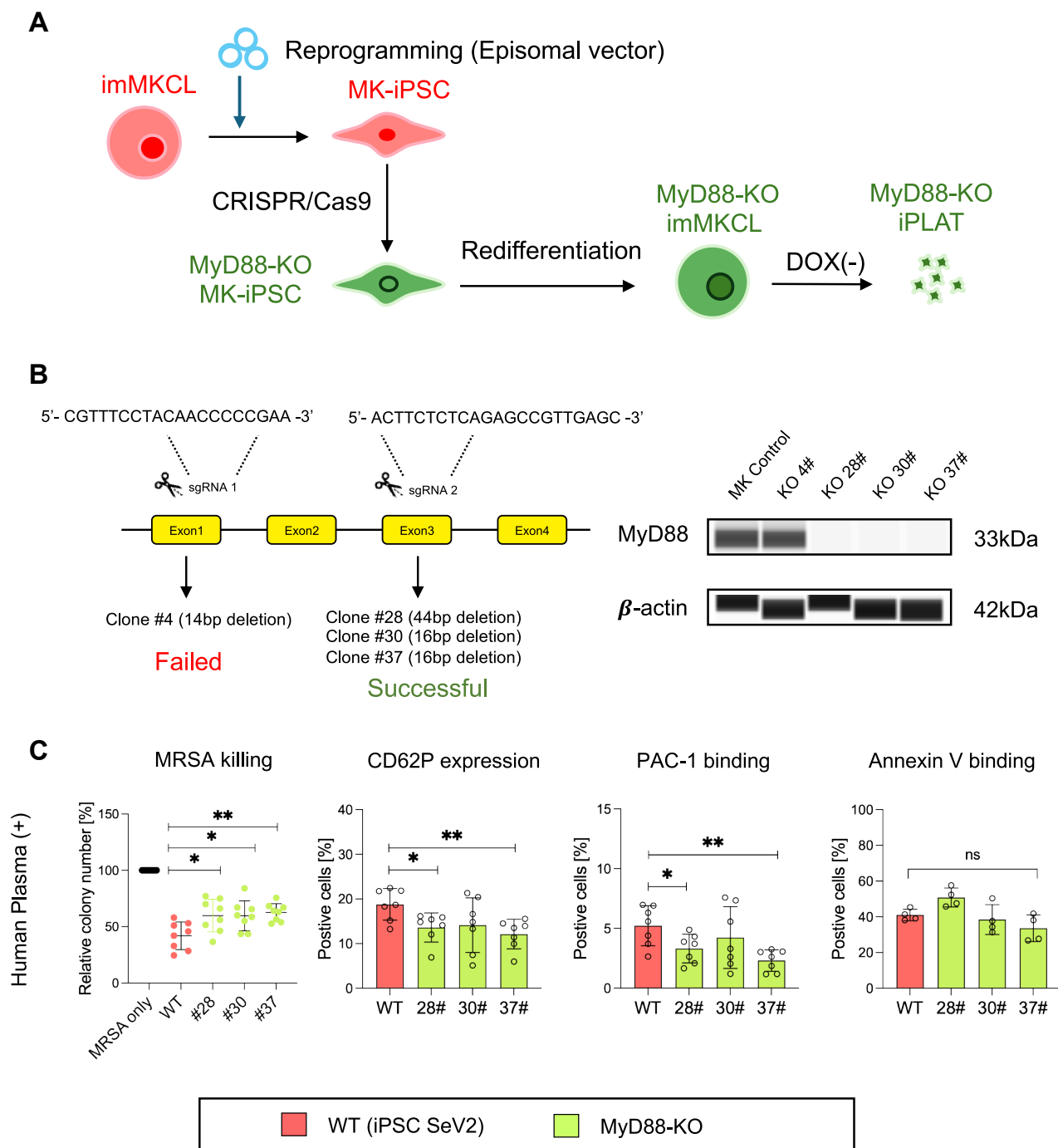


FIGURE 5 MyD88 knockout (KO)-induced pluripotent stem cell (iPSC)-derived platelets (iPSC-PLTs) exhibit attenuated responses to multidrug-resistant *Staphylococcus aureus* (MRSA). (A) Schematic representation of MyD88-KO generation in immortalized megakaryocyte progenitor cell line (imMKCL; Sev2) cells using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system through a re-reprogramming approach. (B) Western blots confirmed that single guide RNA (sgRNA) 1 targeting exon 1 yielded clone 4 and failed in the KO, but sgRNA2 targeting exon 3 yielded clones 28, 30, and 37, which succeeded in the KO. (C) MRSA-killing assay results for wild-type (WT) and MyD88-KO iPSC-PLTs in the presence of human plasma. (D) Cumulative flow cytometry data of CD62P expression, PAC-1 binding, and annexin V binding in WT and MyD88-KO iPSC-PLTs in the presence of human plasma. Compiled data of 3 independent experiments. Data are presented as means $\pm$ SEM. * $P < .05$, ** $P < .01$. DOX, doxycycline; iPLAT, first-in-human clinical trial of iPSC-PLTs; ns, not significant; MK, megakaryocyte.

4 | DISCUSSION

In this study, we confirmed that iPSC-PLTs possess MRSA-killing activity similar to PB-PLTs according to an *in vitro* killing assay. All 3 iPSC-PLT lines showed MRSA-killing capacity similar to or greater than PB-PLTs and suggested the involvement of conventional platelet activation pathways. These observations are in line with a previous study showing that *S aureus* activates platelets via fibronectin and the platelet $\alpha_{IIb}\beta_3$ integrin [16]. Of note, M35-1 iPSC-PLTs showed the highest baseline activation, which may explain their stronger antibacterial activity. Interestingly, transfusion of M35-1 iPSC-PLTs in the iPLAT1 clinical study resulted in a slight elevation of white blood cell counts in the recipient patient [6]. We have also identified immune subsets within imMKCLs [17,18] possibly affecting these immune phenotypes. Notably, our data conflict with a previous study reporting that ticagrelor increases platelet-mediated killing of *S aureus* [2,3]. Alternatively, other mechanisms independent of aggregation may contribute to killing, such as platelet phagocytosis or the trapping of MRSA, with subsequent release of antimicrobial peptides [12–15,19–21]. Furthermore, the outcomes of *in vivo* experiments would be affected by cell types beyond platelets [22,23].

Plasma influenced both bacterial growth and platelet antibacterial activity, likely producing different net effects depending on whether growth promotion or killing predominated. The growth-promoting effect is most likely explained by nutritional supplementation. As for killing, PB-PLTs with high MRSA-killing activity displayed an Ig signature by proteomic profiling (Supplementary Figure 4). Inhibiting IgG binding to Fc γ RIIA with a blocking antibody suppressed killing, supporting a model where IgG binding amplifies bacteria-induced platelet activation through Fc γ RIIA [24]. It is also possible that antibodies against *S aureus* and/or anti- α -toxin contributed significantly to killing. Such a contribution may explain the variability in the killing capacity of PB-PLTs, depending on the antibody titers in the blood donors. In this regard, iPSC-PLTs were comparably uniform, possibly because they were consistently produced using the same pooled human plasma at the Dox-OFF iPSC-PLT production stage. Lastly, although other plasma factors with known antibacterial activity, such as complements and antimicrobial peptides [20,21,25], may also contribute to killing, none were markedly enriched in our proteomic analysis.

Our findings suggest that α -toxin suppresses platelet-mediated killing. One possible explanation for the suppression is α -toxin-induced platelet damage via Ca^{2+} influx, but we did not observe the expected phenotype change (increased annexin V binding). However, it is possible our measurements were not sufficiently sensitive to annexin V binding. It is also possible that an alternative, unidentified mechanism is involved. In addition, the released concentration of α -toxin could affect platelet function without killing. Further studies are required to clarify α -toxin's suppressive effect.

The TLR system has previously been implicated in thrombosis-related platelet activation [26–28] and in crosstalk with innate immune cells [29]. Whether it contributes to direct antibacterial

functions, however, is less clear. Gram-positive bacteria, such as MRSA, contain TLR2 ligands, including PGN, and PB-PLTs are known to express TLR2 [30]. We confirmed that iPSC-PLTs also express TLR2 and can be activated by PGN or the synthetic TLR2 agonist Pam3CSK4 at high concentrations. However, cocubation with MRSA induced only modest activation. To directly probe the role of TLR2 signaling, we generated MyD88-KO iPSC-PLTs using the imMKCL system. Given that MyD88 is an essential adaptor protein for almost all TLR signaling [10], MyD88-KO iPSC-PLTs are an ideal model to clarify the roles of not only TLR2 but also most TLR family molecules in platelets. Notably, MyD88-KO platelets exhibited significantly attenuated MRSA killing, supporting a role for TLR2-MyD88 signaling in this process. However, because killing was not completely abolished, other recognition mechanisms must contribute. Indeed, a previous study reported that platelet activation mediated by PGN-anti-PGN immune complexes can occur through Fc γ RIIA rather than direct TLR2 engagement [31]. Our imMKCL platform offers the opportunity to generate iPSC-PLTs deficient in such candidate molecules, enabling future dissection of additional mechanisms.

Limitations of the present work include variability observed between iPSC-PLTs and PB-PLTs, no assessment of interactions with other immune cells, and the absence of *in vivo* studies. Nevertheless, the observed MRSA-killing capacity of iPSC-PLTs points to translational potential. In particular, human leukocyte antigen class I-deficient iPSC-PLTs could serve as an human leukocyte antigen-independent platform, providing off-the-shelf universal products [9]. Further genetic modification could generate platelets with higher anti-MRSA capacity. For instance, while MRSA activates neuraminidase in platelets, leading to desialylation of platelet surface molecules and eventually compromised circulation [32], neuraminidase-KO iPSC-PLTs could maintain high circulation under severe MRSA infection.

In summary, our findings demonstrate the capacity of iPSC-PLTs to kill MRSA and provide mechanistic insight into the pathways involved. TLR ligands, such as PGN, activate platelets through the TLR2-MyD88 pathway to contribute to the killing of MRSA by platelets by cooperating with plasma components, such as IgG. However, MRSA counters these defenses through α -toxin, displaying another example of the developmental competition between host defense and bacterial pathogenesis. This study provides a basis for applying iPSC-PLTs as a novel therapeutic modality against MRSA-infected patients. The potential to genetically modify iPSC-PLTs promises enhanced MRSA-killing capacity and a useful model to study the mechanisms responsible.

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AUTHOR CONTRIBUTIONS

Q.L. designed and conducted most of the experiments, analyzed the results, and wrote the manuscript. K.N. and I.S. designed and conducted the experiments and analyzed the results. S.N., S.U., M.Y., S.K., and M.N. provided experimental materials and protocols and gave advice. K.S.-I. conducted the soluble C-type lectin-like receptor-2 (sCLEC2) measurement and analyzed the results. V.N. provided experiment materials, protocols, and insights. K.E. cosupervised the project. N.S. supervised the project and wrote the manuscript. All authors reviewed and edited the manuscript.

RELATIONSHIP DISCLOSURE

K.E. is a founder of Megakaryon Co, Ltd, but currently holds no stock. He previously received collaborative research budgets from Megakaryon Co, Ltd, until 2022, and from Otsuka Pharmaceutical Co, Ltd, until 2023. The interests of K.E. have been reviewed and are managed by Kyoto University in accordance with its conflict-of-interest policies.

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SUPPLEMENTARY MATERIAL

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