Valvular Heart Disease

Endocarditis Pathogen Promotes Vegetation Formation by Inducing Intravascular Neutrophil Extracellular Traps Through Activated Platelets

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Background—Endocarditis-inducing streptococci form multilayered biofilms in complex with aggregated platelets on injured heart valves, but the host factors that interconnect and entrap these bacteria-platelet aggregates to promote vegetation formation were unclear.

Methods and Results—In a Streptococcus mutans endocarditis rat model, we identified layers of neutrophil extracellular traps interconnecting and entrapping bacteria-platelet aggregates inside vegetation that could be reduced significantly in size along with diminished colonizing bacteria by prophylaxis with intravascular DNase I alone. The combination of activated platelets and specific immunoglobulin G–adsorbed bacteria are required to induce the formation of neutrophil extracellular traps through multiple activation pathways. Bacteria play key roles in coordinating the signaling through spleen tyrosine kinase, Src family kinases, phosphatidylinositol-3-kinase, and p38 mitogen-activated protein kinase pathways to upregulate the expression of P-selectin in platelets, while inducing reactive oxygen species–dependent citrullination in the arm of neutrophils. Neutrophil extracellular traps in turn serve as the scaffold to further enhance and entrap bacteria-platelet aggregate formation and expansion.

Conclusions—Neutrophil extracellular traps promote and expand vegetation formation through enhancing and entrapping bacteria-platelet aggregates on the injured heart valves. (Circulation. 2015;131:571–581. DOI: 10.1161/CIRCULATIONAHA.114.011432.)

Key Words: blood platelets ■ communicable diseases ■ endocarditis ■ neutrophils

The release of neutrophil extracellular traps (NETs), in addition to phagocytosis, constitutes an important innate effector mechanism to restrain bacterial spreading.1 NETs also exert direct bacteria-killing activity against various gram-positive and gram-negative pathogens through multiple antimicrobial components that adhere to the fibril structures of NETs, such as elastase, myeloperoxidase, cathepsin, and histones.2 Coagulation-generated fibrin barriers may also restrict microbial dissemination, and, interestingly, such activity could be promoted by NETs through tissue factor and factor XII–dependent activation processes that require the proteolytic activity of neutrophil serine protease.3 NET-induced thrombosis prevents the dissemination of Escherichia coli during systemic infection by fostering the compartmentalization of bacteria in liver microvessels and also by restricting bacterial extravasation into surrounding tissue compartments.3 This septic thrombosis, which is also called immunothrombosis, is induced by NETs and other innate immune cells on interaction with invading pathogens and is considered to be an evolutionarily conserved strategy to support innate immune response against pathogen invasion.3,4

Clinical Perspective on p 581

Platelet activation and aggregation following thrombin-induced coagulation activation constitute another key mechanism in thrombosis. Interestingly, platelets also exhibit innate effector characteristics to sense and respond to pathogen-associated molecular patterns such as lipopolysaccharide (LPS).5 Disseminating bacteria or viruses in the bloodstream could be entrapped by intravascular NETs that are frequently encountered in the liver and lung vasculature.6,7 Intravascular NETs in the liver could be induced by LPS-activated platelets and involve Toll-like receptor 4 (TLR4) signaling; however, this accounts for only part of the activation of NETs induced by septic blood from patients.8,9 Thus, it is possible that other activation signals directed through activators present in septic
blood or on activated platelets are required for the induction of intravascular NET formation.

Infective endocarditis (IE) affecting the cardiovascular system is a typical infectious disease caused by disseminating microorganisms into the bloodstream. Blood-borne staphylococci from contaminated medical devices or streptococci from gingival wounds during dental surgery are the most common endocarditis-inducing pathogens that can easily gain access into the bloodstream and colonize the injured heart valves to induce thrombus formation, which is called vegetation.\textsuperscript{8,9} Endocarditis-inducing Staphylococcus aureus or viridans streptococci acquire distinct strategies to avoid immunosurveillance encountered in the bloodstream,\textsuperscript{10,11} and can induce platelet activation and aggregation through distinct mechanisms in a species-specific manner.\textsuperscript{12,13} Electron microscopic analysis has also revealed that bacteria are in complex with platelets inside septic vegetation.\textsuperscript{14} We demonstrated recently that endocarditis pathogens, such as Streptococcus mutans or Streptococcus gordonii, can form biofilms with multilayer architecture that are characterized by bacterial flocs embedded with platelet aggregates in the form of mats or nidi and are refractory to antibiotic prophylaxis.\textsuperscript{15,16} In the plasma, S mutans–induced biofilm formation through platelet aggregate requires anti-bacteria-specific immunoglobulin G (IgG),\textsuperscript{16} a potent opsonin designated for enhancing phagocytosis by neutrophils to clear bacteria in the circulation. However, the reason why neutrophil or intravascular immunity fails to prevent bacterial colonization and biofilm formation on injured heart valves was unclear.

In this study, we present experimental evidence showing that intravascular NETs, designated to control bacteria spreading through the bloodstream during infection, could be hijacked by the endocarditis-inducing pathogen to aid in vegetation formation on injured heart valves. We also delineate the molecular mechanisms underlying bacteria-platelet aggregate–induced NET formation.

**Methods**

**Bacterial Strains and Plasmid**

S mutans GS5 wild-type strain was grown and maintained in brain-heart infusion broth (BHI, Difco Laboratories Inc, Detroit, MI). To generate green fluorescent protein (GFP)–tagged bacteria, S mutans was transformed with the GFPuv sequence containing the shuttle plasmid, pPDGFpuv, which was described in our previous study.\textsuperscript{11}

**Reagents**

Detailed information is available in Methods in the online-only Data Supplement.

**Experimental Streptococcal Endocarditis Rat Model**

A modified rat model of experimental streptococcal endocarditis was performed as previously described.\textsuperscript{16,17} The detailed procedures are available in the online-only Data Supplement Methods.

**Preparation of Platelets and Neutrophils**

This study was approved by the National Taiwan University Hospital Committee for Regulation of Human Specimens and Volunteers, and all human volunteers provided informed consent. Preparation of human platelet-rich plasma (PRP), platelet-poor plasma, washed platelet suspension, and polymorphonuclear leukocytes was performed as previously described.\textsuperscript{11,18} The detailed procedures are available in the online-only Data Supplement Methods.

**In Vitro Bacteria-Platelet Aggregate Formation Assay**

We demonstrated previously that S mutans adopts antibacteria-specific IgG to activate platelets and form biofilm in the plasma.\textsuperscript{16} We defined such bacterial biofilm embedded in aggregated platelets as bacteria-platelet aggregates in the present study. The in vitro bacteria-platelet aggregate formation assay was performed as previously described.\textsuperscript{16} Bacterial biofilm growth was initiated by inoculating individual wells of a 24-well plate with a round glass coverslip with $\approx 10^7$ colony-forming units of bacteria in 200 µL of PRP or platelet-poor plasma supplemented with 1% (wt/vol) glucose. For the induction of NETs and investigation of the role of NETs in promoting and enhancing bacteria-platelet aggregate formation, peripheral blood–isolated neutrophils were added to a final concentration of $2 \times 10^6$ neutrophils/mL. After 16 hours of incubation, the bacteria-platelet aggregates that had formed on the glass coverslip were gently washed with phosphate-buffered saline 3 times and then fixed with 2% paraformaldehyde. After fixation, the bacteria-platelet aggregates were incubated in 0.5% Triton X-100 for 15 minutes and then stained with rhodamine-conjugated phalloidin (1:200 dilution; Invitrogen, Carlsbad, CA) and Hoechst 33258 (Sigma-Aldrich, St. Louis, MO). After washing with phosphate-buffered saline 3 times, the NET formation was observed with a confocal microscope (Leica TCS SP5).

**Induction of NET Formation**

Peripheral blood–isolated neutrophils were placed on the plasma-coated coverslip (100 µL of 10$^5$ cells/mL in Roswell Park Memorial Institute medium) positioned in wells of a 24-well plate and then incubated with 5% CO$_2$ at 37°C for 1 hour. After removing the nonadhered neutrophils, the neutrophils on the coverslip were further stimulated with S mutans (10$^6$ colony-forming units/mL), IgG (1 mg/mL), platelet suspension (10$^5$ platelets/mL), or recombinant P-selectin (1 µg/mL), alone or combined in 300 µL of Roswell Park Memorial Institute medium. In the inhibition assay, the cells were preincubated with neutralizing antibodies or inhibitors, including aspirin (0.5 mg/mL), anti–P-selectin monoclonal antibody (20 µg/mL), anti–P-selectin glycoprotein ligand-1 (PSGL-1) monoclonal antibody (20 µg/mL), anti-CD32 monoclonal antibody (20 µg/mL), anti–Toll-like receptor monoclonal antibody (TLR2; 25 µg/mL), piceatannol (100 µmol/L), PP2 (20 µmol/L), LY294002 (100 µmol/L), or SB203580 (50 µmol/L), or an equal volume of dimethyl sulfoxide as a vehicle control. For the detection of histone citrullination, the mixtures were incubated at 37°C with 5% CO$_2$ for 3 hours. Following the incubations, the cell lysates were prepared in sodium dodecyl sulfate lysis buffer (2% sodium dodecyl sulfate in 62.5 mM Tris, pH 6.8, supplemented with 5% 2-mercaptoethanol and 10% glycerol) and sonicated. The histone citrullination was further analyzed...
by Western blotting with the use of rabbit antibodies against citrullinated histone H3 or histone H3 (1:2000; Abcam, Cambridge, MA). For observations by confocal microscopy, the samples were stained with rhodamine-conjugated phallolidin (1:200 dilution; Invitrogen, Carlsbad, CA) for platelets or Hoechst 33258 for NETs. The NET formation was further observed with a confocal microscope (Leica TCS SP5).

**P-Selectin Expression of Platelets**

The platelet suspension was stimulated with *S* *mutans* (10⁸ colony-forming units/mL) alone or in combination with IgG (1 mg/mL) in the Tyrode solution. In the inhibition assay, the platelets were preincubated with inhibitors, including aspirin (0.5 mg/mL), piceatannol (100 μmol/L), PP2 (20 μmol/L), LY294002 (100 μmol/L), or SB203580 (50 μmol/L), or an equal volume of dimethyl sulfoxide as a vehicle control. After incubation with 5% CO₂ for 3 hours, the P-selectin expression on platelets was further detected by flow cytometry (fluorescent-activated cell sorting; Becton Dickinson, Franklin Lakes, NJ) using a rabbit antibody against human P-selectin (1:100 dilution; eBioscence, San Diego, CA) and fluorescein isothiocyanate–conjugated anti-rabbit IgG antibody (1:500 dilution; Jackson ImmunoResearch labs, West Grove, PA).

For the detection of phospho-p38 mitogen-activated protein kinase (MAPK), the platelet lysate in sodium dodecyl sulfate lysis buffer was analyzed by Western blotting with the use of a rabbit antibody against human phospho-p38 MAPK (1:2000 dilution; Cell Signaling Technology, Danvers, MA).

**Statistical Analysis**

The statistical significance of the difference between 2 sets of data was analyzed by using an unpaired, 2-tailed Student *t* test. Differences between ≥2 sets of data were assessed by using 1-way analysis of variance followed by the Bonferroni multiple-comparisons test. For nonparametrically distributed data, the Mann–Whitney *U* test and the Kruskal–Wallis test with a subsequent Dunn test were used. A *P*<0.05 was considered statistically significant.

**Results**

**NETs Contribute to Vegetation Formation in Situ**

Using GFP-tagged *S* *mutans* GS5 and the rat endocarditis model,15,17 we previously demonstrated that bacterial biofilm embedded in aggregated platelets form a unique layered structure with unidentified interlayers of amorphous but well-organized meshes interconnecting the bacteria-platelet layers inside the vegetation.16 Confocal laser scanning microscopic analysis revealed the presence of extracellular DNA meshes intermixing with the bacteria biofilm inside the vegetation (Figure 1). Immunostaining revealed positive colocalization of elastase, which is a protein from neutrophil primary granules,19 and released DNA meshes, indicating that NETs are present in the DNA meshes (Figure 1).2 Prophylactic administration of DNase I intravenously before infection reduced the DNA meshes, the size of the vegetation, and the number of colonized bacteria in the vegetation (Figure 2A through 2D), and also improved the aortic insufficiency (Figure I in the online-only Data Supplement). DNase I treatment afterward (4 hours postinfection) conducted in parallel experiments also exhibited therapeutic effects on the reduction of both vegetation size and colonizing bacteria (data not shown). DNase I did not affect the growth of *S* *mutans* GS5 in vitro but increased the bacteremia in the infected rats, suggesting that intravascular DNase I did not exert a bactericidal effect on *S* *mutans* (Figure 2E and Figure II in the online-only Data Supplement). Prophylaxis with antibiotics for the prevention of IE is recommended by the American Heart Association,20 but the efficacy remains controversial.21–23 Our previous and current data also showed that prophylaxis with penicillin alone could not completely block vegetation formation because of the deep-seated bacteria-platelet aggregates entrapped in NETs (Figure III in the online-only Data Supplement).19 Prophylactic administration of DNase I together with penicillin significantly reduced the size of the vegetation, the number of colonized bacteria in the vegetation, and the severity of bacteremia (Figure III in the online-only Data Supplement). Together, these results suggested that NETs may contribute directly to the formation of vegetation.

**NETs Increase Bacteria-Platelet Aggregate Formation**

Based on the in vivo observation, we hypothesized that the bacteria-platelet aggregates could induce NET formation, which, in return, promotes the expansion and thickening of...
previously described.16 Interestingly, NET release from purified neutrophils could only be induced by the bacteria-platelet aggregates, but not by the bacteria or ADP-activated platelets alone (Figure 3A, Figures IV, VA, and VB in the online-only Data Supplement), suggesting that the bacteria-platelet aggregate plays a critical role in NET induction. Positive staining for neutrophil elastase further confirmed that the DNA fibers were released from neutrophils (Figure VI in the online-only Data Supplement). Pharmacological inhibition of NADPH oxidase with diphenylene iodonium attenuated NET release, suggesting that the NET formation induced by the bacteria-platelet aggregates was dependent on NADPH oxidase activity (Figure 3B).

It has been shown that NETs can activate platelets directly through histone H3 and H4.24 We found that NETs and histones could further enhance bacteria-platelet aggregate formation when bacteria were cocultured in the PRP based on the quantification of the biomass (Figure 3C and 3D, Figure VIIA and VIIIB in the online-only Data Supplement). NETs or extracellular histones can promote thrombin generation in the PRP.25,26 We also found that NETs or purified histone H4 could enhance thrombin generation, when bacteria were cocultured in the PRP (Figure VIIIC in the online-only Data Supplement). Therefore, activation of both coagulation cascade and platelet through histone H4 may partially account for the bacteria-platelet aggregate formation enhanced by NETs. Such enhancement of bacteria-platelet aggregate formation by NETs could be abolished in the presence of DNase I (Figure 3C and 3D). Taken together, these data support our hypothesis and indicate that bacteria-activated platelets can induce NET formation, which can further enhance bacteria-platelet aggregate formation in the plasma.

**Bacteria Stimulate Neutrophil Citrullination and NET Formation Requires the Combination of Bacteria and Activated Platelets**

To delineate the mechanism by which *S. mutans*-activated platelets induce NET formation, the sequential interaction of neutrophils or platelets with bacteria or each other were examined in a purified system. NET formation was only induced by the bacteria-platelet aggregates (Figure 4A, Figures VC, VD, and VIIIA in the online-only Data Supplement) that formed in response to anti–*S. mutans*-specific IgG (Figure IX in the online-only Data Supplement). NET formation was also observed by using purified rat neutrophils, platelets, and IgG, thus confirming that bacteria-platelet aggregates could induce NET formation in the rat IE experimental model (Figure X in the online-only Data Supplement). Our previous study showed that platelet activation induced by *S. mutans* could be inhibited by aspirin.14 Thus, we assessed the addition of aspirin in this study and found that it significantly inhibited the NET formation in the rat IE experimental model (Figure 4B).

Histone hypercitrullination can mediate chromatin decondensation, which is essential for NET formation.27 We found that *S. mutans* could directly induce neutrophil histone citrullination, which was enhanced when IgG was bridged together with the bacteria (Figure 4C and Figure VIIIB in the online-only Data Supplement) and inhibited by neutralizing these aggregates on the injured heart valves. The role and mechanism of NET formation was further investigated by using in vitro bacteria-platelet aggregate formation assay as...
antibodies against Toll-like receptor 2 (TLR2) or Fc receptor, or an Fc receptor-binding inhibitor (Figure 4D and 4E).

The essential role of TLR2 was further confirmed by using TLR2-deficient neutrophils isolated from TLR2 knockout mice (Figure XI in the online-only Data Supplement). The major pathogen-associated molecular patterns of S mutans (a gram-positive pathogen) for TLR2 recognition are lipoteichoic acid and peptidoglycan, and the receptors for recognizing lipoteichoic acid and peptidoglycan are TLR2 and its signal partners, TLR1 and TLR6.28 Lipoteichoic acid and S mutans peptidoglycan induced histone citrullination in neutrophils, and neutralizing antibodies against TLR1-, TLR2-, and TLR6-inhibited S mutans–induced histone citrullination in neutrophils (Figure XII in the online-only Data Supplement). Stimulation of neutrophil NADPH oxidase may further induce histone citrullination.29 The inhibition of NADPH oxidase by diphenylene iodonium reduced histone citrullination, suggesting that S mutans–induced histone citrullination in neutrophils was dependent on NADPH oxidase activity (Figure 4F). Taken together, these data indicate that S mutans can induce neutrophil histone citrullination through TLR2 and the Fcγ receptor in a NADPH oxidase–dependent manner, and activated platelets are required for promoting NET formation.

Induced P-Selectin Expression on Platelets Involves p38 MAPK Activation

During hemostasis or inflammation, P-selectin that is expressed on activated platelets mediates the initial tethering of circulating neutrophils through PSGL-1.30,31 Neutralizing antibodies against P-selectin or PSGL-1 inhibited the binding of bacteria-platelet aggregates to neutrophils and also blocked NET formation, suggesting that the P-selectin expressed on S mutans–activated platelets mediates NET formation (Figure 5A through 5C). Our previous study indicated that S mutans–mediated platelet activation requires IgG.16 Consistent with this observation, induced P-selectin
expression on S mutans–stimulated platelets also required specific IgG (Figure 5D).

We next investigated downstream signaling pathways of the Fcγ receptor in platelets to identify the mechanisms by which IgG induces P-selectin expression. Pharmacological inhibition of platelets suggested that Src family kinases, Syk, PI3K, and p38 MAPK were involved in IgG-mediated P-selectin expression (Figure 5D). Furthermore, S mutans together with IgG induced the phosphorylation of p38 MAPK in platelets, which was dependent on Src family kinases (Figure 5E). Consistent with these findings, CLMS data showed that pharmacological inhibition of Src family kinases, Syk, PI3K, or p38 MAPK inhibited bacteria-platelet aggregation and NET formation (Figure 5F). Only inhibitors of Src family kinases or Syk, but not aspirin or a P-selectin–neutralizing antibody, could inhibit histone citrullination induced by S mutans–activated platelets (Figure XIII in the online-only Data Supplement). These results confirmed that S mutans can stimulate histone citrullination directly through the bridging of the Fcγ receptor by specific IgG on neutrophils, but not indirectly through activated platelets. Taken together, these data suggest that bacteria stimulate NET formation by upregulating P-selectin on platelets, which involves Src family kinases, Src, PI3K, and p38 MAPK signaling events.

Roles of P-Selectin and IgG in S mutans-Induced NET Formation

The role of P-selectin in stimulating NET formation was further examined in a purified system containing the recombinant P-selectin, bacteria, and IgG. NET release was induced by the combination of bacteria and recombinant P-selectin and was enhanced by the addition of IgG (Figure 6A and 6B). The downstream signaling pathways of PSGL-1 were further investigated for their roles in P-selectin–mediated NET formation. The data from pharmacological inhibition of neutrophils showed that Src family kinases, Syk, Tec kinase Bruton tyrosine kinase, and p38 MAPK, were involved in P-selectin–mediated NET formation (Figure 6C). Taken together, these data suggested that multiple signaling pathways, including those mediated through Fcγ receptor and P-selectin, are involved in NET formation stimulated by S mutans–activated platelets.

Discussion

NETs are recognized as an important link between inflammation and thrombosis in host immune surveillance. However, using a S mutans–induced experimental endocarditis rat model, we have demonstrated a dark side of NETs in vivo. We also demonstrated that the underlying mechanism of NETs formation is well coordinated and controlled by the endocarditis pathogen. Our previous study indicated that on entering the blood stream, IE-inducing pathogens, such as S mutans, can hijack or adopt host innate immune effectors such as IgG or platelets to form biofilms. In the present study, we further demonstrated that NETs formation contributes directly to the expansion of vegetation formation. It is well known that bacterial extracellular DNA contributes to formation of bacterial biofilms. Only NETs and their component, histones, but not bacterial DNA, could trigger subsequent platelet aggregation and coagulation activation (Figure VII in the online-only Data Supplement). In addition to histones, NETs could trap platelets through the von Willebrand factor and activate...
a coagulation system through other pathways, including the direct activation of factor XII or indirect activation of factor XII through elastase and myeloperoxidase. Therefore, NETs, but not bacterial extracellular DNA, contribute directly to the vegetation expansion observed in our IE model.

Based on the results presented in this study, a putative model for the roles of neutrophils and platelets in vegetation maturation and expansion in IE is proposed as follows (Figure 7):

IE-inducing pathogens activate platelets through specific IgG, which contributes to bacteria-platelet aggregate formation on the damaged valve. In addition, the bacteria also activate the infiltrated neutrophils through specific IgG and TLR2 to produce reactive oxygen species, which could further induce histone citrullination that is required for chromatin decondensation. On the other hand, bacteria induce platelet activation and P-selectin expression, which involves Src family kinases, Syk, PI3K, and p38 MAPK signaling. This activation also provides other signals to neutrophils for NET production. The resulting NETs not only induce bacteria-platelet aggregate formation, but also entrap these aggregates to promote vegetation formation. Such a phenomenon may also be applied to the human endocarditis, because NETs have been identified histopathologically in human specimens. Our in vivo study with 9 rats conducted here showed that the digestion of NETs with DNase I significantly reduced the number of bacteria and size of vegetation, which confirmed the role of NETs in the pathogenesis in IE. Therefore, targeting NETs provides a new strategy for controlling IE. Although it has been reported that NETs can kill bacteria, our data showed that the digestion of NETs with DNase I decreased, but not increased, the colonized bacterial number in vivo, suggesting that NETs have a limited effect on bacterial growth. In fact, we found that NETs

Figure 5. IgG-mediated P-selectin expression on platelets involves p38 MAPK activation. Inhibition of the binding of bacteria-platelet aggregates to neutrophils and NET formation without (A) or with the addition of 20 μg/mL of a neutralizing antibody against P-selectin (B) or PSGL-1 (C) in neutrophils stimulated with S. mutans, washed platelets, and IgG. D, P-selectin expression on platelets was further analyzed by flow cytometry. Washed platelet suspensions were pretreated with aspirin (500 μg/mL), the Src family kinase inhibitor PP2 (20 μmol/L), the Syk inhibitor piceatannol (100 μmol/L), the PI3K inhibitor LY294002 (100 μmol/L), or a vehicle control (DMSO) for 30 minutes and then stimulated by S. mutans GS5 (10^7/mL) and IgG (1 mg/mL). The data were analyzed by 1-way ANOVA from a triplicate experiment. **P<0.01. The means±SD from left to right lane was 8.3±0.5, 5.7±0.2, 162.0±19.9, 27.6±5.9, 4.8±0.1, 49.1±6.0, 7.7±0.4, 6.4±0.2, and 146.0±17.7, respectively; n=3. E, Phosphorylation of p38 MAPK in platelets was further analyzed by Western blotting with an antiphospho-p38 MAPK antibody. F, Peripheral blood–isolated neutrophils were stimulated with S. mutans GS5 (10^7/mL), IgG (1 mg/mL), and washed platelets, which were pretreated with the Src family kinase inhibitor PP2 (20 μmol/L), the Syk inhibitor piceatannol (100 μmol/L), the PI3K inhibitor LY294002 (100 μmol/L), or an equal volume of DMSO as a vehicle control. S. mutans were labeled with GFP (green), and the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). NET formation stained with Hoechst 33258 was observed by CLSM (magnification ×630). These experiments were repeated 3 times and a representative experiment is shown. ANOVA indicates analysis of variance; CLSM, confocal laser scanning microscopy; DMSO, dimethyl sulfoxide; DPI, diphenylene iodonium; GFP, green fluorescent protein; IgG, immunoglobulin; MFI, mean fluorescence intensity; NET, neutrophil extracellular trap; p38 MAPK, p38 mitogen-activated protein kinase; PSGL-1, P-selectin glycoprotein ligand-1; and SD, standard deviation.
had a limited effect on *S. mutans* growth in vitro (Figure XIV in the online-only Data Supplement). Taken together, these data suggest that NETs may trap *S. mutans* in platelet aggregates but may not sufficiently kill the bacterium, which is consistent with previous results.36

It was recently proposed that the link between coagulation and the innate immune response may constitute an innate effector mechanism, named immunothrombosis, which mediates the recognition of pathogens and damaged cells and inhibits pathogen dissemination and survival.3 Immunothrombosis is therefore considered to be a physiological process crucial for intravascular immunity, whereas dysregulation of immunothrombosis may be one of the underlying events that trigger thrombotic disorders, such as disseminated intravascular coagulation. Neutrophils constitute a key player in the development of immunothrombosis through the ejection of a meshwork of NETs.1,2 On the other hand, NETs may also provide a scaffold to activate platelets and stimulate thrombus formation.24 Our results provide direct evidence suggesting that NET-mediated immunothrombosis may play an important role in the formation and maturation of vegetation induced by endocarditis pathogen on injured heart valves. Such bacteria-mediated immunothrombosis may also be involved in thromboembolic events, which are serious complications associated with IE in clinics.8,9,16

Research on NETs spans nearly a decade, but the mechanism of NET formation is still unclear and controversial.22,23 NETosis requires reactive oxygen species production, histone citrullination, and chromatin decondensation.3 Reactive oxygen species may trigger Ca2+-dependent PAD4 activity and lead to histone citrullination, which is required for chromatin decondensation in NETosis.27,29 However, stimulation of neutrophils with hydrogen peroxide alone or formyl-methionyl-leucyl-phenylalanine, which is a potent inducer of NADPH oxidase activity, does not induce NETosis,38,39 suggesting that additional processes are required to promote NET formation.37 This may explain why only some groups observed NET formation in response to LPS, but others found that NETosis occurred only in the presence of platelets.22 Our data showed that *S. mutans* could induce histone citrullination in neutrophils in an NADPH oxidase activity–dependent manner, but still required activated platelets to promote NET formation.

The findings of this study indicate that specific IgG plays a key role in *S. mutans*–mediated platelet activation and NET formation. This result is consistent with previous studies showing that platelet activation by IE pathogens requires specific IgG.18,40 However, the underlying downstream signaling pathway of the Fcγ IIa receptor on platelets is not clear. In the presence of IgG, *Streptococcus sanguinis* causes tyrosine phosphorylation of Fcγ IIa, which leads to the phosphorylation of Syk, LAT, and PLCγ2 and was dependent on Src family kinases.41 Our data also showed that *S. mutans*–stimulated P-selectin expression was dependent on Src family kinases and Syk. Furthermore, we also showed that PI3K and p38 MAPK play a role in P-selectin expression on platelets, which might be similar to the signaling pathway of Fcγ in phagocytes.42 Surprisingly, phosphorylation of p38 MAPK was dependent on Src family kinases, but not Syk or PI3K in platelets. These results suggest that the signaling pathways downstream of the Fcγ receptor in phagocytes and platelets are different. It was previously reported that p38 MAP kinase in human platelets could be activated by several platelet agonists, including thrombin, thromboxane A2, ADP, and von Willebrand factor, but the upstream and downstream pathways are not quite similar.43,44 Therefore, the role and the underlying mechanism of p38 MAPK in IgG-mediated platelet activation will require further investigation.

The binding of circulating neutrophils in the bloodstream to activated and immobilized platelets is mainly mediated by the interaction of P-selectin on the activated platelet and PSGL-1
on the neutrophil. The downstream signaling of PSGL-1 could induce integrin activation, including Mac-1 (CD11b/CD18) and lymphocyte function–associated antigen 1 (LFA-1; CD11a/CD18), which contribute to the firm adhesion of neutrophils to immobilized platelets. Studies on sepsis have shown that the inhibition of the adhesion of platelets and neutrophils by targeting LFA-1 reduced NET production. However, LPS could not stimulate P-selectin expression on platelets, and, therefore, the underlying mechanism by which LPS stimulates platelet binding to neutrophils will require further investigation. It is possible that different mechanisms are involved in LPS- and gram-positive pathogen-regulated NETosis. However, neutralizing antibodies against LFA-1 also reduced S. mutans–activated platelet-mediated NETosis (data not shown). Therefore, the contribution of LFA-1 in this process cannot be excluded.

During NETosis induced by bacteria-activated platelets, the same signaling events are required for platelet and neutrophil activation, including Src family kinases and Syk. This could explain why the pharmacological inhibition of these kinases completely inhibited NETosis induced by bacteria-activated platelets (Figure 5F). Nevertheless, the potential adverse effects of these inhibitors may not warrant their routine usage in clinics. In addition to S. mutans, we also investigated the role of NETs in vegetation expansion by using another IE-inducing streptococci, S. gordonii DL1 (Figure XV in the online-only Data Supplement), whose surface proteins Hsa or GspB and SspA or SspB could directly bind to platelets to trigger the platelet activation in an IgG-independent manner. S. gordonii–platelet aggregates also induced NET formation in vitro and in vivo, and prophylactic administration of DNase I combined with penicillin significantly reduced the size of the vegetation and the colonized bacteria in the vegetation, as well (Figure XV in the online-only Data Supplement). Our previous study showed that prophylaxis with aspirin or penicillin could not reduce S. gordonii–induced vegetation formation and bacterial colonization. Therefore, the inclusion of DNase I administration in addition to routine antibiotic agents, such as penicillin, may be helpful in prophylactic therapy for controlling IE.

In summary, we have provided experimental evidence to indicate that endocarditis pathogen could hijack NETs to promote the spreading and thickening of bacterial biofilms accompanied with aggregated platelets leading to the expansion of vegetation.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Infective endocarditis is characterized by the formation of septic thrombus, called vegetation, with embedded pathogens such as viridans streptococci that form biofilms. However, controversy remains on the most effective regimens for either the prophylaxis or treatment of bacterial endocarditis. With the use of a rat endocarditis model, our previous study demonstrated that infective endocarditis–inducing pathogens, such as *Streptococcus mutans* or *Streptococcus gordonii*, could hijack host immune effectors (including antibacterial immunoglobulin G and platelets) to protect phagocytosis and form a biofilm in situ at damaged heart valves. In the present study, we extended these findings and show that streptococci-platelet aggregates can induce neutrophil extracellular trap formation, thus providing the framework necessary for entrapping streptococci-platelet aggregates into well-organized biofilms, and for activating the coagulation system to expand the size of the vegetation, as well. These findings provide sound rationale to support the prophylactic prescription of antiplatelet agents in addition to antibiotics to patients at high risk of endocarditis. However, for those patients requiring dental extraction or other surgical interventions, the disturbance of hemostasis by antiplatelet agents is contraindicated. In this context, we provide direct evidence to indicate that the disruption of neutrophil extracellular traps with agents such as DNase I alone could significantly diminish vegetation formation. Moreover, the concomitant administration of penicillin and DNase I provided a synergistic antimicrobial effect on preventing bacteremia and the formation of vegetation more efficiently. Therefore, targeting neutrophil extracellular traps by agents such as DNase I may provide an efficient alternative to antiplatelet agents for the prophylaxis of endocarditis in patients undergoing surgery in clinics.
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SUPPLEMENTAL MATERIAL

Supplemental methods

Reagents

Human IgG was prepared using the Montage® Antibody Purification Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Recombinant P-selectin was purchased from R&D Systems Inc. (Minneapolis, MN). Aspirin, prostaglandin E1, heparin, the spleen tyrosine kinase (Syk) inhibitor piceatannol, the Src family kinase inhibitor PP2, the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002, the Tec kinase inhibitor LFM-A13, and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 were purchased from Sigma-Aldrich (St. Louis, MO). The neutralizing antibodies, including anti-P-selectin mAb (AK4), anti-PSGL-1 mAb (KPL-1), anti-CD32 mAb (6C4), anti-Toll like receptor 2 mAb (TL2.1) were purchased from eBioscience (San Diego, CA). The Fc receptor binding inhibitor was obtained from eBioscience (San Diego, CA).

Experimental streptococcal endocarditis rat model

Approval for animal use was obtained from the National Taiwan University Institutional Animal Care and Use Committee prior to initiation of experiments. A modified rat model of experimental streptococcal endocarditis was performed as
previously described. Briefly, a polyethylene tube with a stainless steel wire embedded inside was inserted into the left carotid artery above the clavicles and advanced toward the chest to create injury at the aortic valves (9 rats/group). Twenty-four hours following catheter placement, rats were intravenously infected with the bacteria at $1 \times 10^9$ CFU. To analyze the effect of DNase I, 10000 Kunitz units/kg (Sigma-Aldrich, St. Louis, MO) DNase I was injected into the rats 30 min before or 4 h after the inoculation of the bacteria. At 24 h post-infection, the vegetation was harvested. To estimate the number of colonized bacteria, vegetation was removed and then homogenized by ultrasonication, serially diluted in PBS, and plated on BHI to determine colony-forming units per gram of vegetation (CFU/g). The effect of DNase I was evaluated by detecting the size of vegetation and the colonized bacteria density in the vegetation. For confocal laser scanning microscopy (CLSM) analysis, the vegetation harvested from the rat was stained with a rabbit anti-rat elastase antibody (1:50 dilution; eBioscience, San Diego, CA) followed by a rhodamine-labeled anti-rat IgG antibody (1:200 dilution; Jackson ImmunoResearch labs, West Grove, PA). The DNA was further stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO). The NET formation was then observed on a confocal microscope (Leica TCS SP5).
**Preparation of human platelets and PMNs**

Human blood samples were collected from volunteers in the laboratory and added to 3.2% sodium citrate at a ratio of 9:1 by volume. Human platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 150 g for 10 min at room temperature as previously described. The blood that remained after removing the PRP was centrifuged at 630 g for 10 min at room temperature to yield platelet poor plasma (PPP). The washed platelet suspension (PS) was also prepared according to the protocol previously described. PRP was supplemented with prostaglandin E1 (PGE1) (0.5 µM) and heparin (6.4 IU/ml) to stabilize platelets. After centrifugation, the platelets were washed twice with Tyrode solution (136.8 mM NaCl, 2.8 mM KCl, 11.9 mM NaHCO₃, 1.1 mM MgCl₂, 0.33 mM NaH₂PO₄, 1.0 mM CaCl₂, 11.2 mM glucose, and 3.5 mg of bovine serum albumin per ml) with PGE1 (0.5 µM) and heparin (6.4 IU/ml). The preparation was finally resuspended in Tyrode solution at a concentration of 3–5 x 10⁸ platelets/ml. Human PMNs were isolated from heparinized venous blood of healthy individuals using Ficoll-Histopaque (Histopaque 1119 and Histopaque 1083, Sigma-Aldrich, St. Louis, MO).
Supplementary Figure 1. Ultrasonic investigation of aortic insufficiency in the rat IE experimental model. (A) Schematic representation of rat cardiac structure under ultrasonic analysis. (B) The color Doppler-mode analysis of aortic regurgitation was performed by using single element transducer, center frequency 40MHz (Prospect,
S-Sharp Corporation, Taipei, Taiwan). (C) The PW Doppler-mode were sampled at the left ventricular outflow tract. The catheterized Wistar rats were intravenously infected with *S. mutans* GS5 at an inoculum of $10^9$ CFUs without (No treatment) or with (DNase I) the prophylactic administration of DNase I (10,000 Kunitz units/kg).

(D) The severity of aortic regurgitation in No treatment or DNase I was compared by measuring the area of regurgitant flow (after scale normalization) on the PW Doppler-mode analysis. For each rat, the mean of 10 continuous measurements was calculated, and each point represents one rat. The data were analyzed by the Student’s t-test. **$P < 0.01$.** AO, aortic root; LVOT, left ventricular outflow tract; LV, left ventricle; RV right ventricle; LA, left atrium; Ve, vegetation.
Supplemental Figure 2. DNase I has no effect on bacterial growth. An overnight culture of *S. mutans* GS5 (10 µl) was seeded in 1 ml BHI medium with or without the addition of 4 Kunitz units/ml DNase I. The *S. mutans* growth curve was detected by measuring the absorbance at 550 nm.
Supplemental Figure 3. Prophylactic administration of DNase I combined with penicillin significantly reduces the size of the vegetation, the number of colonized bacteria in the vegetation and level of bacteremia. Catheterized Wistar rats were intravenously infected with green fluorescent protein-tagged *S. mutans* GS5 at an inoculum of $10^9$ CFUs. DNase I (10000 Kunitz units/kg), penicillin (600000 units/kg) or DNase I and penicillin in combination was injected into the experimental endocarditis rats 30 min before inoculation of the bacteria. The vegetation formation (A), bacterial colonization inside the vegetation (B), and number of circulating bacteria (C) *in vivo* were further analyzed. Scatter plots show values from individual rat (n=6) and medians (bars). The data were statistically analyzed using
Kruskal-Wallis test with subsequent Dunn’s test; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. 
Supplemental Figure 4. Enlarged figure of Fig. 3A.
Supplemental Figure 5. Measurement of NETs in the supernatant. NET formation was induced by *S. mutans*-platelet aggregates in the PRP. Streptococci were grown in 300 μl PRP or PPP supplemented with 1% (w/v) glucose. Addition of 5 μg/ml ADP in 300 μl PRP was performed as a control. For each well, peripheral blood-isolated neutrophils were added to a final concentration of 2 x 10^6 neutrophils per ml. To detect NETs in the supernatant, the supernatant was harvested. The free DNA was detected by adding Sytox Green at 5 μM and the fluorescence was detected on a
Beckman Coulter PARADIGM™ Microplate Detection Platform (A). The data were analyzed by one-way ANOVA from a triplicate experiment. **P < 0.001. To further extract the NET proteins, DNase I (10 Kunitz units/ml) was added in the supernatant to digest the NETs and the whole cells and the debris were removed by centrifugation. The NET proteins were precipitated with three volumes of cold acetone, and the citrullinated histone H3 was detected by Western blotting (B). Peripheral blood-isolated neutrophils were placed on the plasma-coated coverslip (100 μl of 10^7 cells/ml in RPMI) in wells of a 24-well plate for 1 h. After removing the non-adhered neutrophils, the neutrophils were stimulated with S. mutans GS5 (10^8/ml) alone or in combination with IgG (1 mg/ml) or washed platelets. The supernatants were also harvested for the detection of free DNA (C) and citrullinated histone H3 (D). These experiments were repeated three times and a representative experiment is shown.
Supplemental Figure 6. *In vitro* NET formation stimulated by bacteria-platelet aggregates. Peripheral blood-isolated neutrophils were incubated with *in vitro* *S. mutans*-platelet aggregates. *S. mutans* were labeled with GFP (green) and the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). NET formation was detected by staining with an anti-elastase antibody (1:50 dilution) followed by an Alexa Fluor® 680-conjugated secondary antibody (1:200 dilution) and Hoechst 33258. Visualization was performed on a CLSM (magnification 630 x).
Supplemental Figure 7. Histones promote bacteria-platelet aggregate formation and thrombin generation. (A) *S. mutans* was grown in 300 μl PRP without (left panel) or with (right panel) the addition of 5 μg/ml histone H4. *S. mutans* were labeled with GFP (green) and the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). The bacteria-platelet aggregate formation was observed by CLSM (magnification 630X). (B) The biomass of the bacteria-platelet aggregates represented in (A) was analyzed using the Volocity program. The data were analyzed by Student’s t-test. *P < 0.05. These experiments were repeated three times and a
A representative experiment is shown. (C) Thrombin generation by histones and NET components was analyzed. The NET components were isolated from $2 \times 10^7$ neutrophils/ml stimulated by 100 nM PMA, and the supernatant was harvested after the whole cells and the debris were removed by centrifugation. 5 μg/ml histone H4, 20 μg/ml S. mutans chromosome or 10 μl NET components was added into the 40μl PRP containing $10^8$ /ml S. mutans. The reaction of thrombin generation was initiated by adding 50 μl of reagent SUB (1 mM Z-Gly-Gly-Arg-AMC and 15 mM CaCl₂, Technoclone) and detected at 1-min intervals for 60 min with 360-nm-excitation and 460-nm-emission set in a SpectraMax® M5 reader (Molecular Devices, Sunnyvale, CA, USA). All data were analysed using TGA evaluation software. (Technoclone).
Supplemental Figure 8. A time course of NET formation and histone citrullination stimulated by *S. mutans*-activated platelets. (A) Peripheral
blood-isolated neutrophils were placed on a plasma-coated coverslip (100 μl of 10^7 cells/ml in RPMI) in wells of a 24-well plate for 1 h. After removing the non-adhered neutrophils, the neutrophils were stimulated by *S. mutans* GS5 (10^8/ml) alone or in combination with IgG (1 mg/ml) or washed platelets. *S. mutans* were labeled with GFP (green) and the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). NET formation stained with Hoechst 33258 was observed by CLSM (magnification 630X). A yellow appearance was observed where *S. mutans* formed aggregates with platelets. (B) Histone H3 citrullination in neutrophils stimulated with *S. mutans* GS5, IgG or washed platelets, alone or together, were analyzed by Western blotting. The bottom figure represents the quantification of the data of Western blotting by Image J software. These experiments were repeated three times and a representative experiment is shown.
Supplemental Figure 9. NET formation stimulated by *S. mutans*-activated platelets requires specific IgG. Specific human IgG against *S. mutans* was depleted by incubating purified IgG with *S. mutans*. The IgG that did not bind to *S. mutans* was harvested by centrifugation and filtered. (A) The specific IgG against *S. mutans* was measured by the enzyme-linked immunosorbent assay (ELISA). *S. mutans* GS5 were coated on 96-well polystyrene microtiter plates, and 50 μl normal human IgG or IgG depleted of specific IgG against *S. mutans* (1mg/ml) was added. After incubation, the specific IgG against *S. mutans* was detected by incubation with alkaline phosphatase (AP)-conjugated secondary antibody and AP substrate, and further quantified by measuring the absorbance at 405 nm wavelength. (B) The role of specific IgG against *S. mutans* in NET formation was further elucidated. Peripheral blood-isolated neutrophils were stimulated by *S. mutans* GS5 (10^8 CFU/ml), washed platelets (10^9/ml)
and normal human IgG or IgG depleted of specific IgG against *S. mutans* (1mg/ml), respectively. *S. mutans* were labeled with GFP (green) and the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). The NET formation, stained with Hoechst 33258, was further observed by CLSM (magnification 630 x).
Supplemental Figure 10. Rat NET formation induced by *S. mutans*-platelet aggregates. Peripheral blood-isolated rat neutrophils were placed on the plasma-coated coverslip (100 μl of 10^7 cells/ml in RPMI) in wells of a 24-well plate for 1 h. After removing the non-adhered neutrophils, the neutrophils were stimulated by *S. mutans* GS5 (10^8/ml) in combination with rat IgG (1 mg/ml) and washed rat platelets (10^8/ml). *S. mutans* were labeled with GFP (green) and the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). NET formation stained with Hoechst 33258 was observed by CLSM (magnification 630X).
Supplemental Figure 11. Histone citrullination and NET formation stimulated by *S. mutans* GS5 is dependent on TLR2. Histone citrullination and NET formation in neutrophils isolated from wild-type or TLR2 KO mice were detected. The peripheral blood-isolated neutrophils from wild-type or TLR2 KO mice were stimulated by *S. mutans* GS5 (10^8 CFU/ml), washed platelets (10^8/ml), and mouse serum (containing specific IgG against *S. mutans*). (A) Histone citrullination in neutrophils was detected by Western blot analysis by using rabbit antibodies against citrullinated histone H3 or histone H3. (B) The NET formation was observed by CLSM (magnification 630 x). The *S. mutans* were labeled with GFP (green), the platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution), and the chromosome
DNA was stained with Hoechst 33258.
Supplemental Figure 12. *S. mutans*-induced histone citrullination in neutrophils is depends on PGN and LTA and through TLR1, 2 and 6. Peripheral blood-isolated neutrophils on plasma-coated coverslips were stimulated with *S. mutans* GS5 (10⁸/ml), *S. mutans* PGN (2 μg/ml) or LTA (10 μg/ml). In the inhibition assay, the neutralizing antibody (20 μg/ml) against TLR1, TLR2 or TLR6 was added. These experiments were repeated three times and a representative experiment is shown.
Supplemental Figure 13. Inhibitors of Src family kinases or Syk can inhibit histone citrullination in neutrophils induced by S. mutans-activated platelets.

Peripheral blood-isolated neutrophils on plasma-coated coverslips were stimulated with S. mutans GS5 (10⁵/ml) together with IgG (1 mg/ml) and washed platelets. Aspirin (0.5 mg/ml), or neutralizing antibodies against P-selectin (20 μg/ml) were added and exhibited no effect on histone citrullination stimulated by S. mutans-activated platelet (A). The effect of the Src family kinase inhibitor PP2 (20 μM), the Syk inhibitor piceatannol (100 μM), the PI3K inhibitor LY294002 (100 μM), the Tec kinase inhibitor LFM-A13 (100 μM), the p38 inhibitor SB203580 (50 μM), or a vehicle control (DMSO) was also investigated (B). Only inhibitors of Src family kinases or Syk inhibited histone citrullination induced by S. mutans-activated platelets. These experiments were repeated three times and a representative experiment is shown.
Supplemental Figure 14. NETs exhibit minor effects on *S. mutans* growth.

Neutrophils (10^7 cells/ml) were stimulated with 100 nM PMA for 3 h to release NETs, and then 10^7 CFU/ml *S. mutans* were added. 10 μM cytochalathin D (cyto D) was also added to inhibit phagocytosis and the mixture was incubated at 37°C for 3 h. The samples without neutrophils were performed as a control. Data are presented as a growth ratio of *S. mutans* versus the bacterial seeding numbers. Statistical analysis was performed using one-way ANOVA. ns, not significant. These experiments were repeated three times and a representative experiment is shown.
Supplemental Figure 15. NETs formation induced by S. gordonii DL1 and prophylactic effect of DNase I combined with penicillin. (A) Catheterized Wistar rats were intravenously infected with green fluorescent protein-tagged S. gordonii DL1 at an inoculum of $10^9$ CFUs. The vegetations were then harvested and stained with Hoechst 33258. The in situ NET formation inside the vegetation was observed by confocal microscopy (magnification 630X). (B) S. gordonii DL1 was grown in 300 μl PRP, and peripheral blood-isolated neutrophils were added to a final concentration of $2 \times 10^6$ neutrophils per ml. S. gordonii DL1 were labeled with GFP (green) and the
platelets were visualized by staining with rhodamine-conjugated phalloidin (1:200 dilution). The NET formation stained with Hoechst 33258 was observed by CLSM (magnification 630X). Catheterized Wistar rats were intravenously infected with green fluorescent protein-tagged *S. gordonii* DL1 at an inoculum of $10^9$ CFUs. DNase I (10000 Kunitz units/kg) or DNase I combined with penicillin (600000 units/kg) was injected into the experimental endocarditis rats 30 min before inoculation of the bacteria. The vegetation formation (C) and bacterial colonization inside the vegetation (D) were further analyzed. Scatter plots show values from individual rat (n=5) and medians (bars). The data were statistically analyzed using Kruskal-Wallis test with subsequent Dunn’s test: * $P < 0.05$. Results indicated that NETs were also found in *S. gordonii* DL1-induced endocarditis and DNase I combined with penicillin could also effectively reduce both vegetation size and colonized bacteria.
Reference

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