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

Running title: Jung et al.; NETs promote vegetation formation

Chiau-Jing Jung, PhD1; Chiou-Yueh Yeh, PhD2; Ron-Bin Hsu, MD3; Chii-Ming, Lee, MD4; Chia-Tung Shun, MD5; Jean-San Chia, PhD1,2

1Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan; 2Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan; 3Dept of Surgery, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan; 4Dept of Cardiology, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan; 5Dept of Forensic Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan

Address for Correspondence:
Jean-San Chia, PhD
Graduate Institute of Immunology, College of Medicine
National Taiwan University
No. 1 Jen Ai Road Section 1
Taipei, 10051, Taiwan, ROC
Tel: 886-2-23123456 ext 88222
Fax: 886-2-23926238
E-mail: chiajs@ntu.edu.tw

Abstract

Background—Endocarditis-inducing streptococci form multi-layered biofilms in complex with aggregated platelets on injured heart valves. But 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 (NETs) 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 IgG-adsorbed bacteria are required to induce the formation of NETs 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 up-regulate the expression of P-selectin in platelets, while inducing reactive oxygen species-dependent citrullination in the arm of neutrophils. NETs in turn serve as the scaffold to further enhance and entrap bacteria-platelet aggregate formation and expansion.

Conclusions—NETs promote and expand vegetation formation through enhancing and entrapping bacteria-platelet aggregates on the injured heart valves.

Key words: infective endocarditis, infectious disease, platelet, neutrophil extracellular traps
Introduction

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 -negative pathogens through multiple antimicrobial components that adhere to the fibril structures of NETs, such as elastase, myeloperoxidase (MPO), 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.\(^3,4\) This septic thrombosis, which is also called immunothrombosis, is induced by NETs and other innate immune cells upon interaction with invading pathogens and is considered to be an evolutionarily conserved strategy to support innate immune response against pathogen invasion.\(^3,4\)

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 TLR4 signaling; however, this only accounts for only part of the activation of NETs induced by septic blood from patients.\(^4,5\) 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 bloodstream and colonize the injured heart valves to induce thrombus formation, which is called vegetation.\textsuperscript{8,9} Endocarditis-inducing \textit{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 \textit{Streptococcus mutans} or \textit{Streptococcus gordonii}, can form biofilms with multilayer architecture that are characterized by bacterial floes embedded with platelet aggregates in the form of mats or nidi and are refractory to antibiotic prophylaxis.\textsuperscript{15,16} In the plasma, \textit{S. mutans}-induced biofilm formation through platelet aggregation required anti-bacteria specific IgG,\textsuperscript{16} a potent opsonin designated for enhancing phagocytosis by neutrophils in order to clear bacteria in the circulation. But 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 bacterial 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. 11

Reagents

Detailed information is available in SUPPLEMENTAL MATERIAL.

Experimental streptococcal endocarditis rat model

A modified rat model of experimental streptococcal endocarditis was performed as previously described. 16, 17 The detailed procedures are available in the SUPPLEMENTAL MATERIAL.

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 (PPP), washed platelet suspension (PS) and PMNs was performed as previously described. 11, 18 The detailed procedures are available in the SUPPLEMENTAL MATERIAL.

*In vitro* bacteria-platelet aggregate formation assay

We demonstrated previously that *S. mutans* adopts anti-bacteria specific IgG to activate platelets and form biofilm in the plasma. 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 approximately $10^7$ CFU bacteria in 200 μl PRP or PPP supplemented with 1% (w/v) 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 per ml. After 16 h incubation, the bacteria-platelet aggregates that had formed on the glass coverslip was gently washed with PBS three times and then fixed with 2% paraformaldehyde. After fixation, the bacteria-platelet aggregates were incubated in 0.5% Triton X-100 for 15 min and then stained with rhodamine-conjugated phalloidin (1:200 dilution; Invitrogen, Carlsbad, CA) and Hoechst 33258 (Sigma-Aldrich, St. Louis, MO). After washing with PBS three times, the NET formation was observed with a confocal microscope (Leica TCS SP5).

\textbf{Induction of NET formation}

Peripheral blood-isolated neutrophils were placed on the plasma-coated coverslip (100 μl of $10^7$ cells/ml in RPMI) positioned in wells of a 24-well plate and then incubated with 5% CO\textsubscript{2} at 37°C for 1 h. After removing the non-adhered neutrophils, the neutrophils on the coverslip were further stimulated with \textit{S. mutans} (10\textsuperscript{8} CFU/ml), IgG (1 mg/ml), PS (10\textsuperscript{8} platelets/ml) or recombinant-P-selectin (1 μg/ml), alone or combined in 300 μl RPMI medium. In the inhibition assay, the cells were pre-incubated with neutralizing antibodies or inhibitors, including aspirin (0.5 mg/ml), anti-P-selectin mAb (20 μg/ml), anti-PSGL-1 mAb (20 μg/ml), anti-CD32 mAb (20 μg/ml), anti-Toll like receptor mAb (TLR2) (25 μg/ml), piceatannol (100 μM), PP2 (20 μM), LY294002 (100 μM), LFM-A13 (100 μM), or SB203580 (50 μM), or an equal volume of DMSO as a vehicle control. For the detection of histone citrullination, the mixtures were incubated at
37°C with 5% CO₂ for 3 h. Following the incubations, the cell lysates were prepared in SDS lysis buffer (2% SDS 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 using 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 phalloidin (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 PS was stimulated with *S. mutans* (10⁸ CFU/ml) alone or in combination with IgG (1mg/ml) in the Tyrode solution. In the inhibition assay, the platelets were pre-incubated with inhibitors, including aspirin (0.5 mg/ml), piceatannol (100 μM), PP2 (20 μM), LY294002 (100 μM), or SB203580 (50 μM), or an equal volume of DMSO as a vehicle control. After incubation with 5% at 37°C CO₂ for 3 h, the P-selectin expression on platelets was further detected by flow cytometry (FACS; Becton Dickinson, Franklin Lakes, NJ, USA) using a rabbit antibody against human P-selectin (1:100 dilution; eBioscience, San Diego, CA) and FITC-conjugated anti-rabbit IgG antibody (1:500 dilution; Jackson ImmunoResearch labs, West Grove, PA). For the detection of phospho-p38 MAPK, the platelet lysate in SDS lysis buffer was analyzed by Western blotting using 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 two sets of data was analyzed by using an unpaired, two-tailed Student’s t-test. Differences between more than two sets of data were
assessed using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple-comparisons test. For nonparametrically distributed data, Mann-Whitney U test and Kruskal-Wallis test with subsequent Dunn’s 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, 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. 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 co-localization of elastase, which is a protein from neutrophil primary granules, and released DNA meshes, indicating that NETs are present in the DNA meshes (Figure 1). 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, 2B, 2C and 2D), and also improve the aortic insufficiency (Supplemental Figure 1). DNase I treatment afterwards (4 h post-infection) 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 intra-vascular DNase I did not exert a bactericidal effect on *S. mutans* (Figure 2E and Supplemental Figure 2). 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 due to the deep-seated bacteria-platelet aggregates entrapped in NETs (Supplemental Figure 3).¹⁶ 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 (Supplemental Figure 3). 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 expansion and thickening of these aggregates on the injured heart valves. The role and mechanism of NET formation was further investigated using *in vitro* bacteria-platelet aggregate formation assay as 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, Supplemental Figure 4, 5A, and 5B), suggesting that the bacteria-platelet aggregate play a critical role in NET induction. Positive staining for neutrophil elastase further confirmed that the DNA fibers were released from neutrophils (Supplemental Figure 6). Pharmacological inhibition of NADPH oxidase with diphenylene iodonium (DPI) 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 co-cultured in the PRP based on the quantification of the biomass (Figure 3C, 3D, Supplemental Figure 7A and 7B). NETs or extracellular histones can promote
thrombin generation in the PRP. We also found that NETs or purified histone H4 could enhance thrombin generation, when bacteria were co-cultured in the PRP (Supplemental Figure 7C). 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, Supplemental Figure 5C, 5D, and 8A) that formed in response to anti-*S. mutans* specific IgG (Supplemental Figure 9). NET formation was also observed using purified rat neutrophils, platelets, and IgG, thus confirming that bacteria-platelet aggregates could induce NET formation in the rat IE experimental model (Supplemental Figure 10). Our previous study showed that platelet activation induced by *S. mutans* could be inhibited by aspirin. Thus, we assessed the addition of aspirin in this study and found that it inhibited significantly the NET formation, further confirming that NET formation requires bacteria-activated platelets (Figure 4B).

Histone hypercitrullination can mediate chromatin decondensation, which is essential for NET formation. 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
Supplemental Figure 8B) and inhibited by neutralizing antibodies against Toll-like receptor 2 (TLR2) or Fcγ receptor, or an Fc receptor binding inhibitor (Figure 4D and 4E). The essential role of TLR2 was further confirmed using TLR2-deficient neutrophils isolated from TLR2 knockout mice (Supplemental Figure 11). The major pathogen-associated molecular patterns (PAMPs) of S. mutans (a gram positive pathogen) for TLR2 recognition are lipoteichoic acid (LTA) and peptidoglycan (PGN), and the receptors for recognizing LTA and PGN are TLR2 and its signal partners, TLR1 and TLR6.28 LTA and S. mutans PGN induced histone citrullination in neutrophils, and neutralizing antibodies against TLR1, 2, and 6 inhibited S. mutans-induced histone citrullination in neutrophils (Supplemental Figure 12). Stimulation of neutrophil NADPH oxidase may further induce histone citrullination.29 Inhibition of NADPH oxidase by DPI 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, 5B and 5C). Our previous study indicated that S. mutans-mediated platelet activation requires IgG.18 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 in order 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 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 (Supplemental Figure 13). 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 up-regulating 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 upon 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 its component, histones, but not bacterial DNA, could trigger subsequent platelet aggregation and coagulation activation (Supplemental Figure 7). In addition to histones, NETs could trap platelets through von Willebrand factor and activate 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 contributes directly to 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 ROS, 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 phenomenon may also be applied to the human endocarditis, because NETs have been identified histophathologicaly in human specimens. Our in vivo study with nine rats conducted here showed that 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 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 had a limited effect on S. mutans growth in vitro (Supplemental Figure 14). 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.

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. Immunothrombosis is therefore considered to be a physiological process crucial for intravascular immunity, while dysregulation of immunothrombosis may be one of the underlying events that triggers thrombotic disorders, such as disseminated intravascular coagulation. Neuphils constitute a key player in the development of immunothrombosis.
through the ejection of a meshwork of NETs.\textsuperscript{1,2} On the other hand, NETs may also provide a scaffold to activate platelets and stimulate thrombus formation.\textsuperscript{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.\textsuperscript{8,9,16}

Research on NETs spans nearly a decade, but the mechanism of NET formation is still unclear and controversial.\textsuperscript{22,37} NETosis requires ROS production, histone citrullination, and chromatin decondensation.\textsuperscript{37} ROS may trigger Ca\textsuperscript{2+}-dependent PAD4 activity and lead to histone citrullination, which is required for chromatin decondensation in NETosis.\textsuperscript{27,29} However, stimulation of neutrophils with hydrogen peroxide alone or formyl-methionyl-leucyl-phenylalanine (fMLP), which is a potent inducer of NADPH oxidase activity, does not induce NETosis,\textsuperscript{38,39} suggesting that additional processes are required to promote NET formation.\textsuperscript{37} This may explain why only some groups observed NET formation in response to LPS while others found that NETosis occurred only in the present of platelets.\textsuperscript{2,5} Our data showed that \textit{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 \textit{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.\textsuperscript{18,40} However, the underlying downstream signaling pathway of the Fc\textgamma IIA receptor on platelets is not clear. In the presence of IgG, \textit{S. sanguinis} causes tyrosine phosphorylation of Fc\textgamma IIA, which leads to the phosphorylation of Syk, LAT, and PLC\gamma2 and were dependent on Src family kinases.\textsuperscript{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. 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 (TxA2), ADP, and von Willebrand factor, but the upstream and downstream pathways are not quite similar. 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 LFA-1 (CD11a/CD18), which contribute to the firm adhesion of neutrophils to immobilized platelets. Studies on sepsis have shown that 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 pathogens-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 (Supplemental Figure 15), 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 as well as the colonized bacteria in the vegetation (Supplemental Figure 15). 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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Conflict of Interest Disclosures: None.

References:


Figure Legends:

Figure 1. *In situ* NET formation inside vegetation on the damaged valve in bacterial endocarditis rats. Catheterized Wistar rats were intravenously infected with green fluorescent protein-tagged *S. mutans* GS5 at an inoculum of $10^9$ CFUs. The vegetations were then harvested and stained with Hoechst 33258 and anti-elastase antibody (1:50 dilution) followed by a Texas red-conjugated secondary antibody (1:200 dilution). The co-localization of elastase and extracellular DNA indicate the *in situ* NET formation inside the vegetation as observed by confocal microscopy (magnification 630X). The bottom panels represent projections of z-stacks. The experiments were repeated three times and a representative experiment is shown.

Figure 2. Digestion of NETs by DNase I reduces the bacterial colonization and vegetation size *in vivo*. (A) DNase I (10000 Kunitz units/kg) was injected into the experimental endocarditis rats 30 min before inoculation of the bacteria. The top images show vegetation formation on the damaged valve *in situ*. The vegetation that formed 24 h after injection with DNase I was smaller in size. The bottom images are the CLSM images inside the vegetation. Bars indicate 10 μm. (B) The NETs inside the vegetation was quantified with a V olocity program from three separate experiments. Colocalization of elastase and extracellular DNA was defined as NETs. The data were analyzed by the Student’s t-test. **$P < 0.01$. The mean ± SD was 33.9 ± 7.3 in no treatment group versus 8.4 ± 1.3 in DNase I group; n=3. The effects of DNase I in the vegetation formation (C), bacterial colonization (D), and number of circulating bacteria (E) *in vivo* were further analyzed for statistical significance. Scatter plots show values from individual rat (n=9) and medians (bars). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ as determined by Mann-Whitney *U* test. The mean ± SD and median were 4.2 ± 1.9 and 4.0 in no treatment group versus 1.4 ± 1.3 and
0.9 in DNase I group in panel C. The mean log_{10} CFU ± SD and median were 7.5 ± 0.7 and 7.5 in no treatment group versus 5.4 ± 1.1 and 5.4 in DNase I group in panel D. The mean log_{10} CFU ± SD and median were 1.9 ± 0.3 and 1.8 in no treatment group versus 2.4 ± 0.6 and 2.5 in DNase I group in panel E.

**Figure 3.** NETs contribute to bacteria-platelet aggregate formation in the PRP. (A) *S. mutans* was grown in 300 µl PRP (left panel) or PPP supplemented with 1% (w/v) glucose (middle panel). Addition of 5 µg/ml ADP in 300 µl PRP was performed as a control (right panel). For each well, peripheral blood-isolated neutrophils were added to a final concentration of 2 x 10^6 neutrophils per ml. *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 observed by CLSM (magnification 630X). NETs were only induced by bacteria-platelet aggregates in the PRP. (B) The NADPH oxidase inhibitor DPI (10 µM) was added when neutrophils were added in the bacteria-platelet aggregates in the PRP, and visualization was performed on a CLSM (magnification 630X). (C) 3D-CLSM image of *S. mutans*-platelet aggregates in PRP, PRP containing isolated neutrophils, or PRP containing isolated neutrophils and 2 Kunitz units/ml DNase I. (D) The biomass of the bacteria-platelet aggregates represented in (C) was analyzed using the Volocity program. The mean ± SD was 0.66 ± 0.07 in control group, 1.21 ± 0.15 in neutrophil group, and 0.49 ± 0.07 in neutrophil+DNase I group; n=4. The data were analyzed by one-way ANOVA. ***P < 0.001. These experiments were repeated three times and a representative experiment is shown.

**Figure 4.** Bacteria stimulate histone citrullination in neutrophils and activated platelets are required for promoting NET formation. (A) Peripheral blood-isolated neutrophils were placed on
the plasma-coated coverslip (100 μl of 10⁷ 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 (10⁸/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 is observed where *S. mutans* formed aggregates with platelets. NETs could only be induced by bacteria-platelet aggregates. (B) Addition of aspirin (500 μg/ml) inhibited the bacteria forming aggregates with platelets and also inhibited NET formation. (C) Histone H3 citrullination in neutrophils stimulated by *S. mutans* GS5, IgG, or washed platelets, alone or together, were analyzed by Western blotting. The role of IgG, TLR2, or NADPH oxidase activity in histone H3 citrullination in neutrophils was further confirmed by incubating the samples with a neutralizing antibody against FcR (20 μg/ml) or Fc binding inhibitor (D), neutralizing antibody against TLR2 (25 μg/ml) (E), or NADPH oxidase inhibitor, DPI (10 μM) (F).

**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 μM), the Syk inhibitor piceatannol (100 μM), the PI3K inhibitor LY294002 (100 μM), the p38 inhibitor SB203580 (50 μM), or a vehicle control (DMSO) for 30 min and then stimulated by *S. mutans* GS5 (10⁸/ml) and IgG (1 mg/ml). The data were analyzed by one-way ANOVA from a triplicate
experiment. ***P < 0.001. The mean ± SD from left to light 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 anti-phospho-p38 MAPK antibody. (F) Peripheral blood-isolated neutrophils were stimulated with *S. mutans* GS5 (10^8/ml), IgG (1 mg/ml), and washed platelets, which were pretreated with the Src family kinase inhibitor PP2 (20 μM), the Syk inhibitor piceatannol (100 μM), the PI3K inhibitor LY294002 (100 μM), the p38 inhibitor SB203580 (50 μM), 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 630X). These experiments were repeated three times and a representative experiment is shown.

**Figure 6.** The roles of P-selectin and IgG in *S. mutans* GS5-stimulated NET formation.

Peripheral blood-isolated neutrophils were stimulated by *S. mutans* GS5 (10^8/ml) combined with recombinant P-selectin (1 μg/ml) (A) or *S. mutans* GS5 (10^8/ml) combined with recombinant P-selectin (1 μg/ml) and IgG (1 mg/ml) (B). *S. mutans* were labeled with GFP (green) and NET formation stained with Hoechst 33258 was observed by CLSM (magnification 630X). (C) Peripheral blood-isolated neutrophils were pretreated with the Src family kinase inhibitor PP2 (20 μM), the Syk inhibitor piceatannol (100 μM), the Tec kinase inhibitor LFM-A13 (100 μM), the p38 inhibitor SB203580 (50 μM), or an equal volume of DMSO as a vehicle control for 30 min, and then stimulated with *S. mutans* GS5 (10^8/ml) in combination with recombinant P-selectin (1 μg/ml) and IgG (1 mg/ml) for 3 h. NET formations were stained with Hoechst 33258. The area of NET DNA fibers and total DNA was quantified from three random fields per sample using the Volocity program. Total DNA was taken as 100%. The data were analyzed by one-way
ANOVA from a triplicate experiment. *P < 0.05; ***P < 0.001. The mean ± SD from left to light lane was 5.9 ± 1.8, 5.4 ± 1.6, 23.4 ± 5.8, 37.9 ± 6.1, 3.0 ± 1.0, 4.7 ± 2.5, 22.9 ± 3.7, 5.7 ± 2.5 and 36.9 ± 7.9, respectively; n=3. These experiments were repeated three times and a representative experiment is shown.

**Figure 7.** A hypothetical model for NET-induced vegetation expansion on injured heart valves. IE pathogens form aggregates with platelets on the heart valve by stimulating platelets through IgG bearing the Fcγ receptor. After activation, P-selectin is expressed on the platelets, which involves Src family kinases, Syk, PI3K, and p38 MAPK signaling pathways. In addition, the bacteria stimulate ROS production and histone citrullination in neutrophils through the Fcγ receptor and TLR2. Two separate signals from the bacteria and the activated platelets, respectively, eventually induce NETosis. The resulting NETs not only promote coagulation activation and platelet aggregation but also entrap bacteria-platelet aggregates *in situ* to promote and consolidate vegetation expansion.
Figure 1