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Interactions of periodontal pathogens with platelets in the gingival crevicular fluid of patients with periodontitis

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Abstract

Aim: To explore the immunological defensive effects of platelets on periodontal pathogens in the gingival crevicular fluid (GCF).

Materials and Methods: GCF samples were collected from 20 patients with periodontitis and 10 healthy controls. Platelets in the GCF were detected by immunocytochemistry and immunofluorescence. Isolated platelets from healthy volunteers were co-cultured with *Porphyromonas gingivalis* (*Pg*) and *Fusobacterium nucleatum* (*Fn*). The interactions between platelets and periodontal pathogens were observed by transmission and scanning electron microscopy. The isolated platelets plus neutrophils were co-cultured with *Pg* or *Fn*, and the formation of neutrophil extracellular traps (NETs) was evaluated by staining with Sytox Green.

Results: The platelet level in the GCF was higher in patients with periodontitis than in healthy controls. Platelets interacted with bacteria and neutrophils in the GCF. In vitro, platelets recruited and engulfed periodontal pathogens. In response to periodontal pathogens, neutrophils released web chromatin, and platelets promoted the formation of intensive NETs.

Conclusions: Platelets, migrating to the gingival sulcus, may exert direct antibacterial effects or assist neutrophils.

KEYWORDS
gingival crevicular fluid, neutrophil, periodontal pathogen, periodontitis, platelet

Clinical Relevance

Scientific rationale for study: Previous studies demonstrated platelet involvement in the host response to periodontal infection in periodontal tissues; however, platelet participation in host defences against periodontal pathogens in the gingival crevicular fluid (GCF) remains unclear.

Principal finding: Platelets interacted with bacteria and neutrophils in the GCF. Platelets engulfed and recruited periodontal pathogens and promoted the formation of neutrophil extracellular traps (NETs).

Practical implications: Platelets play a primary immunological role by directly or indirectly inhibiting periodontal pathogens. Therefore, platelet agents have the potential for use as topical antimicrobials in the prevention or treatment of periodontitis.

Huanxin Meng and Yalin Zhan contributed equally to this work.

1 | INTRODUCTION

Periodontitis is a microbial dysbiosis-associated inflammatory disease, characterized by gingival tissue inflammation and periodontal destruction. Dental plaque is the initial event leading to periodontitis. Exploring the association between microbe and host immune system contributes to a deeper understanding of the pathogenesis of periodontitis and the development of strategies for periodontitis prevention and treatment.

As the colonization site for microorganisms, the gingival sulcus is the frontline of resistance to periodontal pathogenic bacteria. Previous studies on gingival crevicular fluid (GCF) have focused mainly on neutrophils. Following their activation by periodontal pathogens or their virulence factors, leukocytes migrate to the gingival sulcus. Among these leukocytes, about 95% are polymorphonuclear neutrophils (Delima & Van Dyke, 2003). Neutrophils in the GCF are the first line of defence against periodontal pathogens through degranulation, phagocytosis, reactive oxygen species, and neutrophil extracellular traps (NETs) (Mantovani et al., 2011).

Platelets are small cell fragments. In addition to their well-known roles in haemostasis and thrombosis, platelets also participate in the immune inflammation response. Platelets have also recently been studied in periodontitis. Previous work in our group showed that platelets aggregate with neutrophils in the gingival tissue of patients with generalized aggressive periodontitis, suggesting that platelets play a crucial role in the immune inflammation response in periodontal tissues (Zhan et al., 2017). A recent study reported higher platelet numbers and more platelet-associated active factor, namely platelet factor 4 (PF4) in the GCF of patients with periodontitis compared with healthy controls (Brousseau-Nault et al., 2017). However, the specific role of platelets in the GCF remains unclear.

Recent studies have observed that platelets can resist microbial infection (Palankar et al., 2018; Koupenova et al., 2019). Platelets interact with bacteria through direct or indirect binding. Various bacterial components bind to platelet glycoprotein IIb–IIIa indirectly via fibrinogen and fibronectin or directly (Hamzeh-Cognasse et al., 2015). In addition to binding fibrinogen, serine–aspartate repeat protein G from *Staphylococcus epidermidis*, can also directly interact with platelet glycoproteins (Brennan et al., 2009).

NETosis is a unique method of neutrophil death, in which nuclear chromatin is decondensed and long chromatin filaments are released into the extracellular space. Web DNA is decorated with granular proteins. NETs have antimicrobial functions in that they trap and kill pathogens in blood and tissues (Brinkmann, 2004; Brinkmann & Zychlinsky, 2007). NETs occur in purulent crevicular exudates and supragingival plaque (Vitkov et al., 2009). Neutrophils stimulated by *Porphyromonas gingivalis* (*Pg*), *Fusobacterium nucleatum* (*Fn*), and *Streptococcus gordonii* are more significantly associated with NET structures compared with unstimulated neutrophils (White et al., 2014; Hirschfeld et al., 2017). Furthermore, activated platelets induce the formation of NETs in transfusion-related acute lung injury (TRALI) and septic blood (Clark et al., 2007; Caudrillier et al., 2012).

Thus, we assumed that platelets might directly participate in the defence response to periodontal pathogens or act synergistically with neutrophils in the GCF.

Therefore, the purpose of this study was to explore the role of platelets in the first line of defence (GCF) against periodontal pathogens.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Ethical approval for this study was obtained from the Ethics Committee of Peking University Health Science Center (PKUSSIRB-201627026) and all subjects provided informed consent prior to their enrolment.

2.2 | Patient selection

Patients with periodontitis and healthy volunteers were recruited from the Department of Periodontology of Peking University School and Hospital of Stomatology from December 2019 to December 2020. After excluding subjects with a history of systemic diseases, anti-microbial medications, anti-inflammatory drugs, or periodontal treatment within the last 6 months, 20 periodontitis patients and 10 healthy controls were finally selected.

2.3 | GCF collection and processing

Supragingival plaques were removed in the selected sites, which were isolated with cotton rolls and dried with an air syringe to prevent saliva contamination. A sterile filter paper strip (2 mm × 10 mm, Whatman, UK) was gently inserted into the gingival crevice above the base of the gingival groove and remained for 30 s, avoiding mechanical irritation. Strips contaminated with blood were discarded. Four strips from four sites per patient or healthy controls were pooled in a 1.5 -ml sterile Eppendorf tube containing 400 μl of phosphate-buffered saline (PBS) (pH 7.4, Hyclone) and protease inhibitor cocktail (50:1, Huaxingbio). The GCF samples were stored at 4°C and processed within 24 h. The tube was vibrated on a Vortex mixer at 1.5 A for 5 min, and the diluent was processed by Cytospin at 1000 rpm for 3 min. The smears were fixed in 4% paraformaldehyde (Solarbio) for 15 min. They were then used for Wright-Giemsa staining to exclude blood pollution and immunological staining for platelet observation.

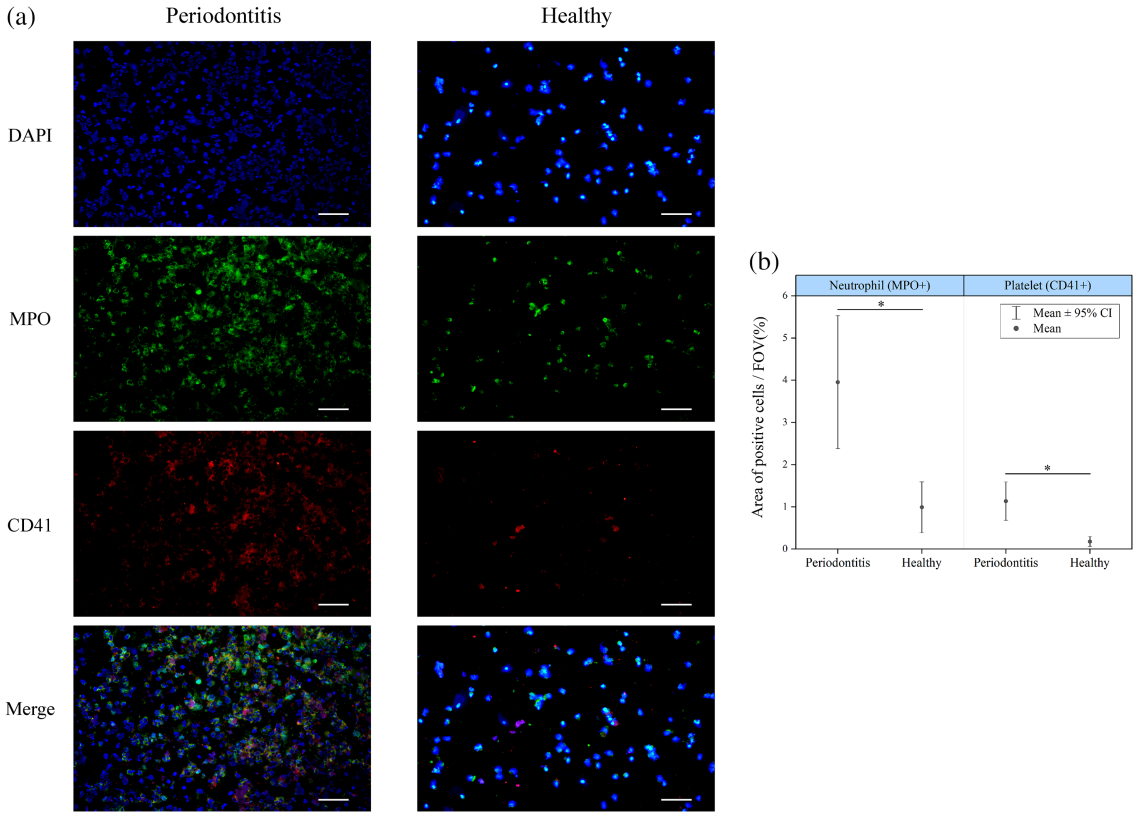

FIGURE 1 Platelets interact with neutrophils in the gingival crevicular fluid of inflammatory sites. Representative images show immunofluorescence staining of DNA (blue), neutrophils (green), and platelets (red). More platelets are visible in the gingival crevicular fluid of patients with periodontitis than in that of healthy controls. Patients with periodontitis also have more neutrophils in the gingival crevicular fluid than healthy individuals. Platelets gathered with neutrophils in the gingival crevicular fluid (GCF) samples of periodontitis patients. Scale bar, 100 μm (a). (b) Quantification of MPO^+ and $CD41^+$ in smears of the GCF of patients with periodontitis ($n = 20$) and healthy controls ($n = 10$). Six fields were randomly selected for the quantitative study of every GCF sample. The area of positive cells/fields of view (FOV) (%) is the percentage of MPO^+ or $CD41^+$ staining area in FOVs. The analyses of immunofluorescence staining were performed using ImageJ analysis software. Error bar, mean ± 95% confidence interval. *$p < .05$, relative to the healthy control group, based on *t*-test

2.4 | Immunofluorescence

The smears were blocked with 10% goat serum and then incubated overnight at 4°C with the following primary antibodies: anti-CD41 (1:100, rabbit monoclonal antibody, ab134131, Abcam) and anti-MPO (1:100, mouse monoclonal antibody, ab8216, Abcam). They were then incubated with secondary antibodies: Alexa-594 anti-rabbit (1:200, Zsbio) and Alexa-488 anti-mouse (1:200, Zsbio) for 1 h in darkness at 37°C. The nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were obtained by confocal microscopy (LSM 710, Zeiss, Germany).

2.5 | Immunocytochemistry

To prevent non-specific binding, the GCF smears were blocked with 10% goat serum (Zsbio, China) for 30 min and then incubated with rabbit monoclonal anti-CD41 (1:100, ab134131, Abcam) overnight at 4°C, followed by incubation with goat anti-rabbit IgG (Zsbio, China) for 30 min at 37°C, before staining with 3,3′-diaminobenzidine (DAB) (Zsbio, China) for 3 min. The nuclei were counter-stained with haematoxylin.

2.6 | Platelet isolation

Human venous blood was drawn from healthy volunteer donors aged 20–40 years. Washed platelets were isolated from acid citrate dextrose anti-coagulated blood from 10 adult healthy volunteers. The citrated blood was spun at 200*g* for 15 min to obtain platelet-rich plasma that was subsequently mixed with 100 nM PGE1 (Sigma-Aldrich) and spun at 700*g* for 15 min. The washed platelets were resuspended in RPMI 1640 medium (Procell).

2.7 | Bacterial strains

The in vitro studies used two representative strains of *Pg* ATCC 33277 and W83, and *Fn* typical strain ATCC 25586 stored at the

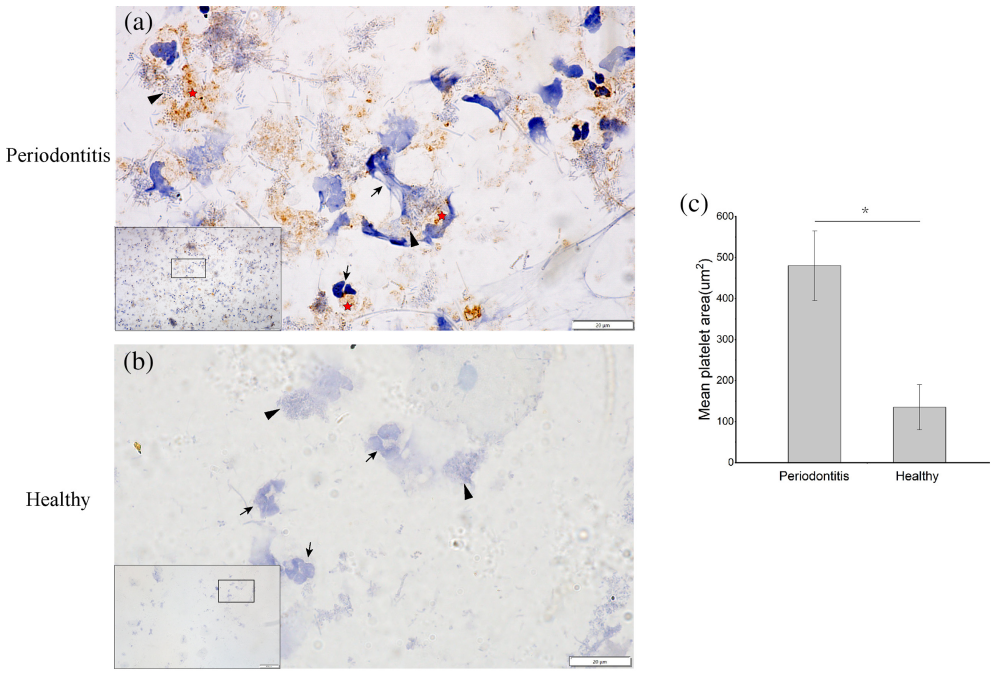

FIGURE 2 Platelets interact with bacteria and neutrophils in the gingival crevicular fluid (a–f). Representative immunocytochemistry images show that more platelets ($CD41^+$, brown, pentacle), bacteria (blue cluster, triangle), and neutrophils (blue lobulated nucleus or reticular chromatin, arrow) are present in the gingival crevicular fluid of patients with periodontitis (a) than healthy subjects (b). Neutrophils can be identified by their characteristic lobulated nucleus. Bacteria are mostly small and spherical or rod-shaped and about 1 μm in size. Bacterial clumps intertwine with platelets and are captured by neutrophil extracellular traps. Scale bars: 20 μm (a, b) and 100 μm (the lower left of a and b). (c) Histogram comparing the relative recruitment of platelets in the gingival crevicular fluid of patients with periodontitis and healthy controls. For each smear, the platelet area was counted in 10 fields of view. Periodontitis group ($n = 20$), Healthy group ($n = 10$) (data are expressed as the means ± SD; $^*p < .01$, *t*-test)

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2.8 | Transmission electron microscopy observation

Washed platelets were mixed with *Pg* ATCC 33277, *Pg* W83, and *Fn* ATCC 25586 (MOI = 10). After co-cultivation at 37°C and 5% CO_2 for 30 min, the samples were centrifuged at 10,000*g* for 10 min. The sediments were fixed with 2.5% glutaraldehyde and post-fixed in 1% osmic acid, dehydrated with gradient alcohol, replaced with propylene oxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a JEM-1400 electron microscope.

2.9 | Scanning electron microscopy observation

Washed platelets were mixed with *Pg* 33277, *Pg* W83, and *Fn* 25586 (MOI = 10). After 30-min co-cultivation at 37°C and 5% CO_2, the samples were fixed with 2.5% glutaraldehyde and then washed, fixed, dehydrated, and sputter-coated with AuPd thin films for scanning electron microscopy (Hitachi SU8010, Japan) analysis.

2.10 | Detection of NETs

EDTA anticoagulant venous blood, collected from normal adult volunteers, was used to isolate neutrophils using Polymorphprep (Solario) according to the manufacturer's protocol. Neutrophil purity was approximately 90%. The culture medium was RPMI 1640 plus 1% fetal bovine serum. Red blood cells were lysed in red blood lysis buffer. After co-cultivation of neutrophils (1×10^5/ml) and periodontal pathogens (neutrophil:bacteria = 1:100) with or without platelets (1×10^6/ml) for 1 h at 37°C and 5% CO_2, these cells were incubated with rabbit monoclonal anti-H3Cit (1:200, ab134131, Abcam) overnight at 4°C, followed by incubation with goat anti-rabbit IgG (Zsbio) for 30 min. The chromatin was stained with Sytox Green Nucleic Acid Stain (Thermo Fisher Scientific, UK) and observed by fluorescence microscopy (Olympus, BX53).

3 | RESULTS

3.1 | Platelets interacted with bacteria and neutrophils in the GCF

GCF samples from 20 patients with periodontitis and 10 healthy controls were collected, smeared, and stained. Confocal laser-scanning microscopy showed more neutrophils in the periodontitis samples

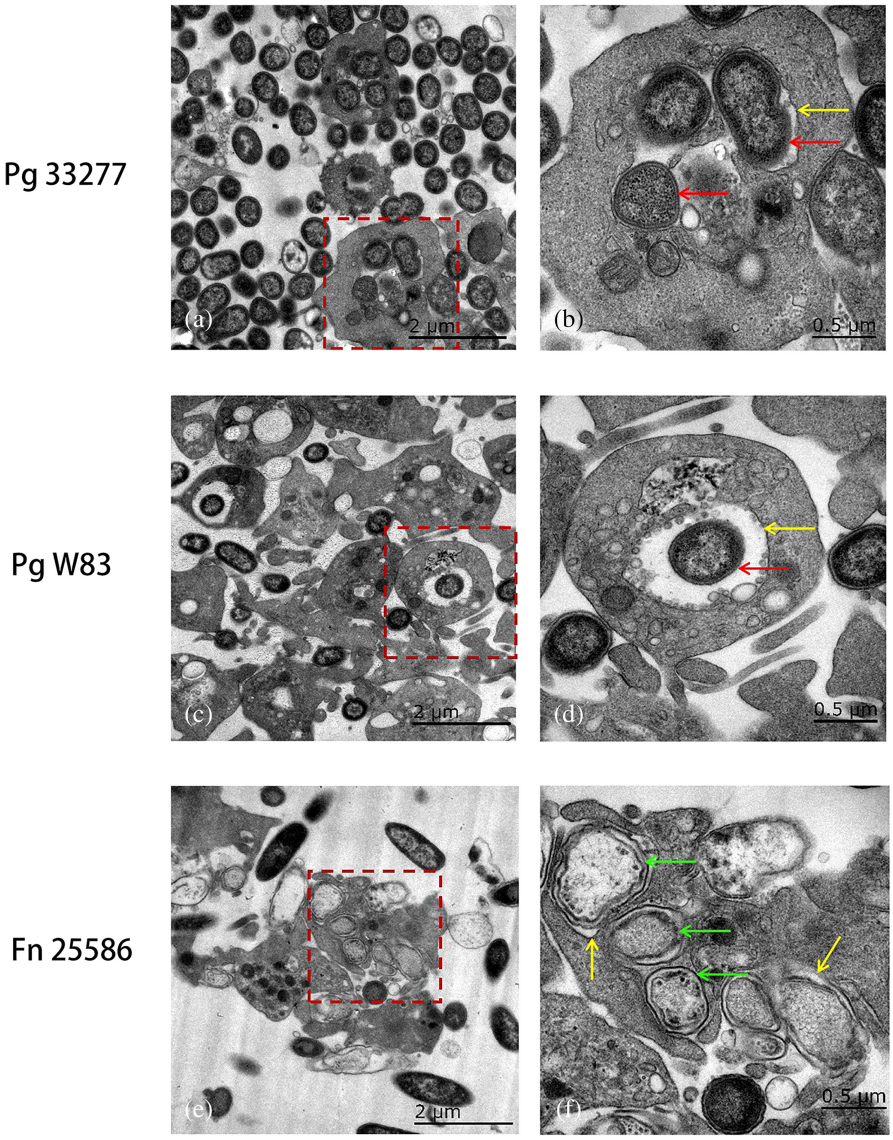

FIGURE 3 Platelets internalize periodontal pathogens. Transmission electron micrographs showing platelet phagocytosis of *Porphyromonas gingivalis* 33277 (a and b, red arrows), and *P. gingivalis* W83 (b and c, red arrows). For *Fusobacterium nucleatum* (e and f), the platelet engulfed its vesicle (f, green arrows). The limiting membranes (b, d, and f, yellow arrows) of the engulfing vacuoles surround the internalized bacteria (b and d) or vesicle (f). Scale bar: 2 μm (a, c, e) and 0.5 μm (b, d, f)

compared with the healthy controls. Moreover, large numbers of platelets aggregated with neutrophils were observed in the GCF of patients with periodontitis (Figure 1).

Immunocytochemistry showed cells and bacteria in the GCF clearly and easily. As shown in Figure 2 and Figure S1, an abundance of primarily rod-shaped or bulbiform bacteria was observed in the GCF. The bacterial clumps were associated with aggregated platelets in the inflammatory sites. Most neutrophil nuclei had lost their primary condensed state and were swollen. The escaped chromatin encircled bacteria–platelet complexes. Some platelets were closely attached to neutrophils to form neutrophil–platelet complexes.

3.2 | Platelets engulfed and aggregated periodontal pathogens

Platelets and neutrophils were isolated from the venous blood of 10 healthy volunteers. The washed platelets were first co-cultivated with the periodontal pathogens *Pg* 33277, *Pg* W83, and *Fn* 25586. As shown in Figure 3, transmission electron microscopy revealed that *Pg* 33277 and W83 were phagocytosed and the limiting membrane of vacuoles (engulfing vacuoles) surrounded the internalized bacteria. Vesicles secreted by *Fn* 25586 were contained in the platelet cytoplasm, with platelet pseudopods encircling the bacteria.

Next, we examined the aggregation of platelets and bacteria. As shown in Figure 4, dispersive *Pg* 33277 and *Pg* W83 did not gather, while Fn aggregated spontaneously. When incubated with platelets, platelet pseudopods captured and aggregated *Pg* 33277. However, *Pg* W83 and *Fn* aggregated on spreading platelets.

3.3 | Platelets promoted the formation of NETs in response to periodontal pathogens

Co-culture of isolated platelets, neutrophils, and periodontal pathogens showed that *Pg* 33277, *Pg* W83, and *Fn* 25586 induced the formation of NETs. More NETs and intense chromatin webs were observed when in co-culture with platelets (Figure 5).

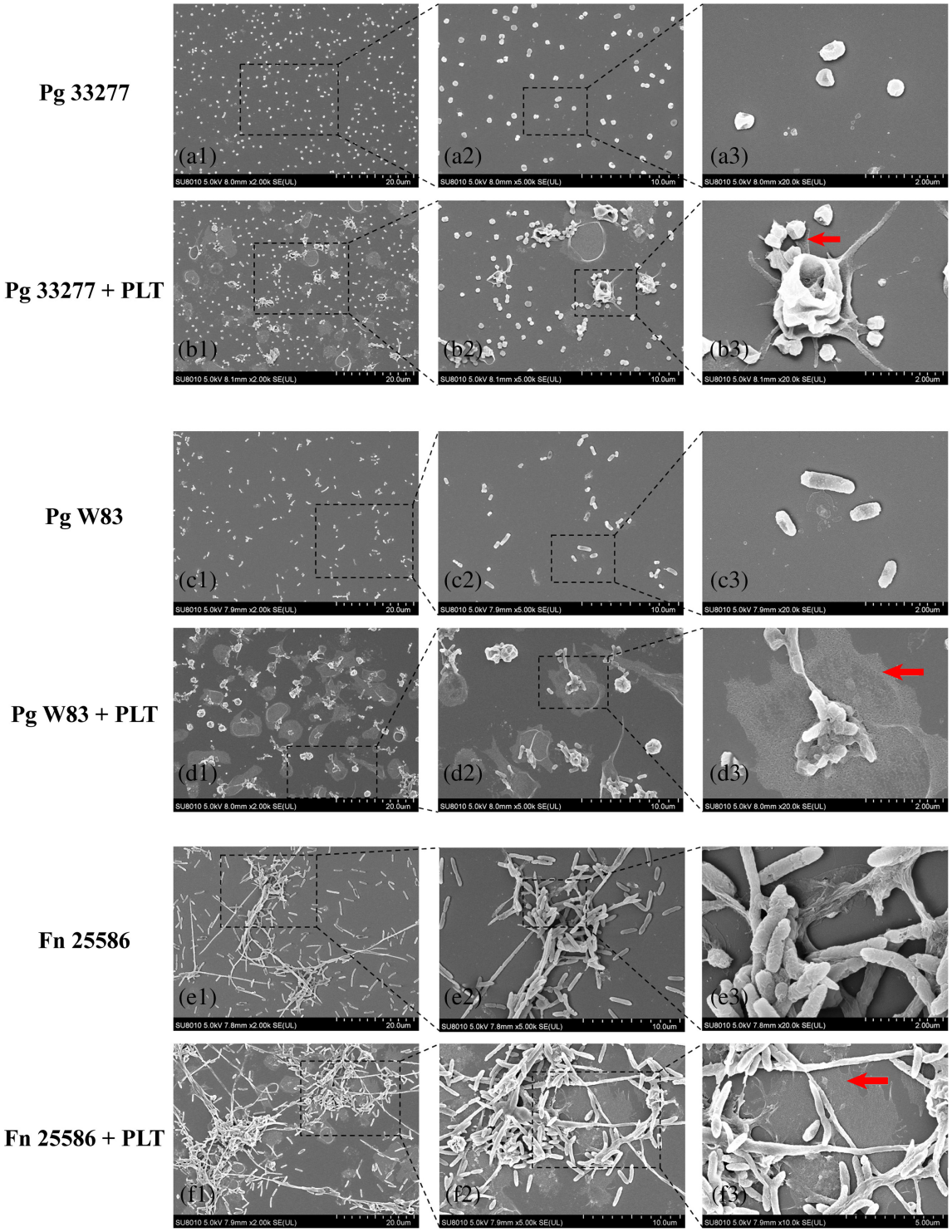

FIGURE 4 Platelets promote the aggregation of periodontal pathogens. Scanning electron microscope images showing that *Porphyromonas gingivalis* were scattered (a, c), while *Fusobacterium nucleatum* aggregated spontaneously (e). For *Pg* 33277, platelets produced protruded pseudopodia (b3, red arrow) to seize the pathogen. For *Pg* W83, the platelets spread (d3, red arrow), with *Pg* W83 masses sitting on the large platelets. In co-culture with platelets, *Fn* clumps were attached to spreading platelets (f3, red arrow)

4 | DISCUSSION

The results of this study revealed the presence of platelets in the GCF of both healthy subjects and patients with periodontitis, with higher levels exhibited in the patients group. Platelet interactions with bacteria and neutrophils were also observed. More specifically, the platelets and bacteria were intertwined, and the platelets adhered closely to the neutrophils (Figure 6a). Based on our observations of both host

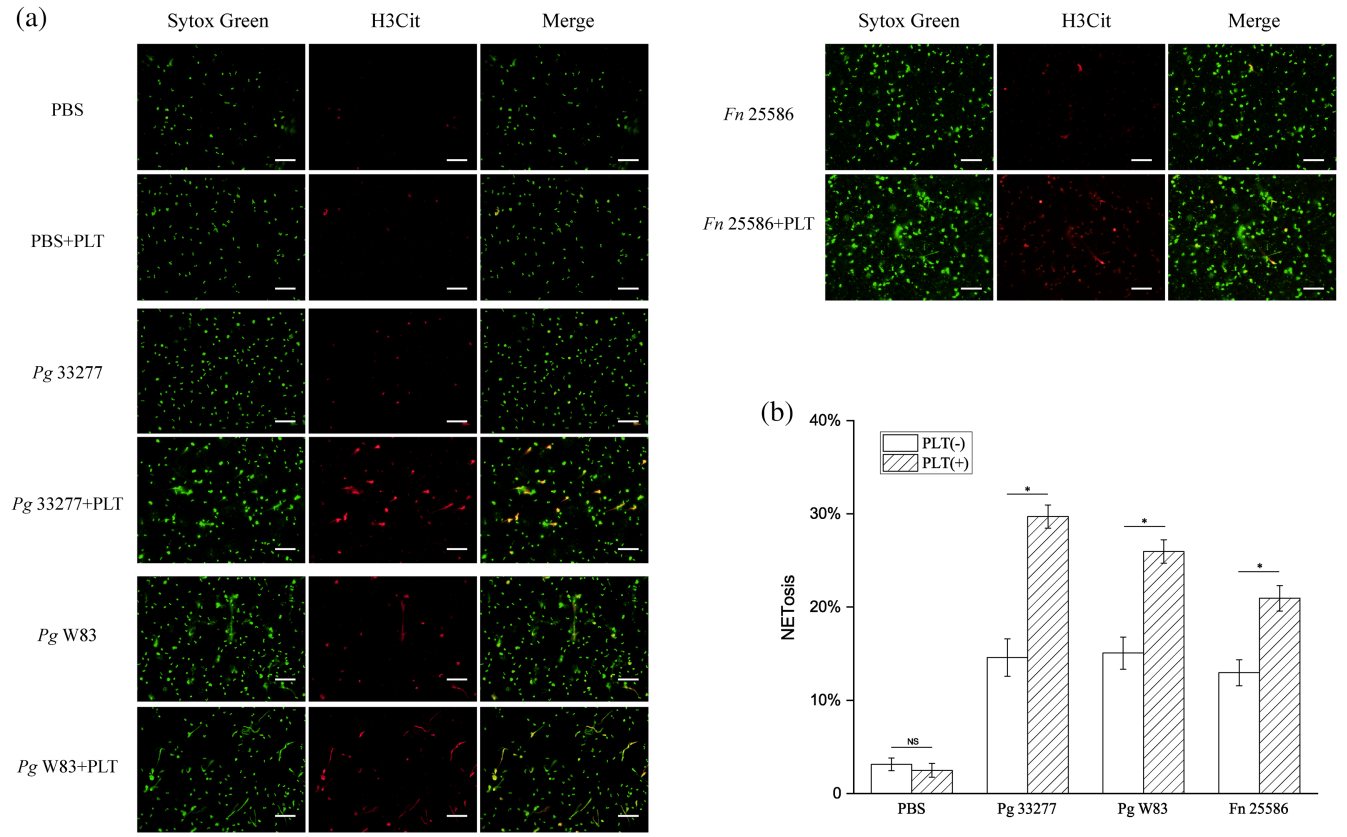

FIGURE 5 Platelets promote the formation of neutrophil extracellular traps (NETs) in human neutrophils in response to exposure to *Pg* 33277, *Pg* W83, and *Fn* 25586. (a) Representative images from the fluorescence staining of DNA (green) and H3Cit (red) showed no NET formation following treatment with PBS or platelets alone. Neutrophils treated with *Pg* 33277, *Pg* W83, or *Fn* 25586 alone produced fewer NETs compared to those treated with platelets and *Pg* 33277, *Pg* W83, and *Fn* 25586. Scale bar: 100 μm (a). (b) Bar chart showing the percentage of neutrophils forming NETs (mean ± SD) co-cultured with periodontal pathogens lacking or supplemented with platelets. The data presented from the platelets and neutrophils of three healthy volunteers. Six fields were randomly selected for each sample to quantify NETs. $^*p < .05$ compared with the group without platelets, based on *t*-test

cells and bacteria in the GCF, we speculate that platelets may directly interact with bacteria, or may assist neutrophils in killing bacteria through NETs. We then showed that washed platelets engulfed and recruited periodontal pathogens. Additionally, isolated platelets enhanced the formation of NETs (Figure 6b). The results of these in vitro studies confirmed our hypothesis.

The GCF samples were excluded from blood contamination through Giemsa staining. We speculate that platelets undergo an active migration process like that of neutrophils. In the allergic asthma model, lung histology revealed that platelets migrate out of vessels and localize underneath the airways (Pitchford et al., 2008). In vitro, driven by stromal cell-derived factor 1, platelets transmigrate through a transwell membrane and endothelium (Kraemer et al., 2010). This evidence shows that platelets can migrate. However, further research is needed to determine how platelets move out of the gingival blood vessels to reach the gingival sulcus.

Our previous work confirmed that *Pg* promoted platelet–neutrophil aggregates (Zhan et al., 2016). In this study, many platelets adhered to neutrophils and formed platelet–neutrophil aggregates in the GCF of patients with periodontitis. These results suggest that periodontal pathogens or their violence factors may induce platelet–neutrophil aggregates in the GCF.

Pg is a keystone pathogen of chronic periodontitis (Mysak et al., 2014). W83 and ATCC 33277 are classic high and low virulent strains of *Pg*, respectively. *Fn* is a numerically dominant pathogen in plaque biofilm (Signat et al., 2011). Therefore, the present study used two types of *Pg* and *Fn*.

The results of these in vitro studies have demonstrated that platelets could engulf and recruit periodontal pathogens (*Pg* 33277, *Pg* W83, *Fn* 25586). Thus, platelets can act directly on periodontal pathogens. The phagocytosis and recruitment of platelets in response to periodontal pathogens restrain their activities to some extent. HIV particles are reportedly internalized by activated platelets, and the particles were present in the engulfing vacuoles. Several α-granules then fuse, and their fibrinogen content is released into the vacuoles (Youssefian et al., 2002). *Pg* has also been detected in the engulfment vacuoles of aggregated platelets (Li et al., 2008). Platelets contain three distinct types of granules, α-granules, dense granules, and lysosomes. The most abundant type of granule is the α-granule. Activated platelets release many bioactive molecules, such as PF4,

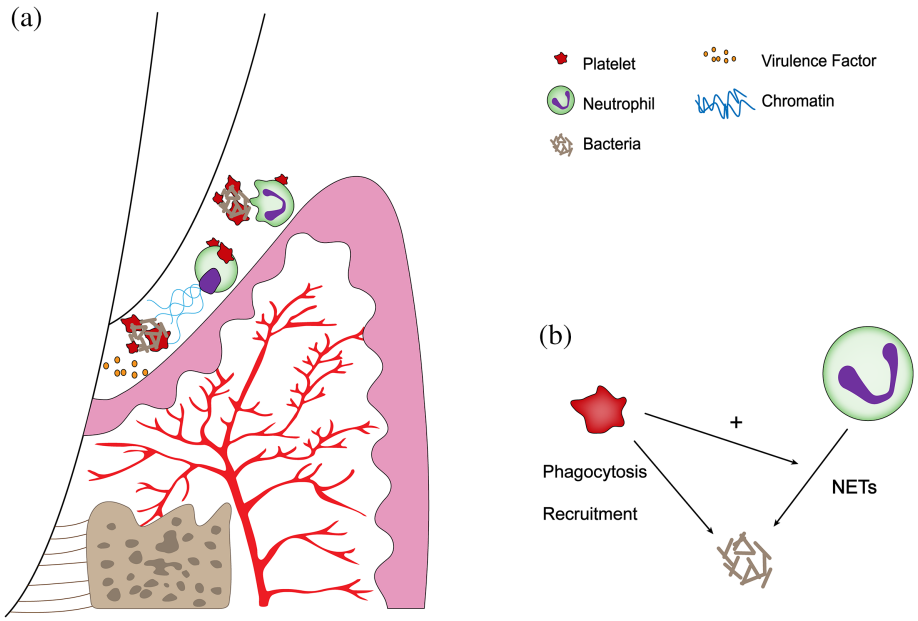

FIGURE 6 (a) Proposed model showing platelets in the gingival crevicular fluid, interacting with bacteria and neutrophils. (b) Platelets exert immunological effects by recruiting and engulfing periodontal bacteria or promoting the formation of neutrophil extracellular traps (NETs)

β-thromboglobulin, and HMGB1 (Brandt et al., 2000; Zhou et al., 2018). Platelets have direct antimicrobial effects that are mediated via the degranulation and release of antimicrobial peptides such as PF4 and CCL5 (Yeaman, 2014). The evidence suggests that platelets may digest periodontal bacteria after engulfment.

We observed that platelets gathered periodontal pathogens, indicating that the bacterial aggregation observed in the GCF may be induced by platelets. To a certain extent, it may limit the movement of periodontal pathogens, which is one aspect that platelets play direct bacteriostatic roles. These results are partly consistent with a study published in *Cell* (Gaertner et al., 2017), which reported that platelets collected and bundled *Escherichia coli*, so that these platelet-bound bacterial bundles were resistant to shear stress, facilitating neutrophil activation in sepsis. In addition, the platelets extended pseudopods to capture and gather *Pg* 33277. In *Pg* W83 and *Fn* 25586, the bacterial clumps aggregated on the spreading platelets. These results suggest that platelets responds differently to different periodontal pathogens.

We further observed that the platelets enhanced the neutrophilic response to periodontal pathogens by promoting the formation of NETs. This finding suggests that platelets can indirectly kill bacteria by interacting with neutrophils. Early studies have shown that platelet TLR4 activation induced binding to adherent neutrophils in the sinusoids and promoted neutrophil activation and the formation of NETs (Clark et al., 2007). NETs are produced through P-selectin-PSGL-1-mediated platelet–neutrophil interactions (Etulain et al., 2015). However, the detailed mechanisms of the interactions between platelets and neutrophils are not fully understood and need to be further investigated.

The results of this study provide evidence of the presence of platelets in the GCF. To our knowledge, this is the first report that platelets, associated with bacteria and neutrophils, take part in immune defence response in the GCF. However, this study has several limitations. Many platelets in the GCF were gathered with bacteria clusters and the boundaries of the platelets were unclear. Thus, platelet levels can only be assessed through the area of the specific staining rather than the platelet count. Further exploration is required to determine whether the platelet response induces periodontal destruction, and how the interactions between platelets and neutrophils may be regulated to resist periodontal pathogens.

5 | CONCLUSION

Overall, there were many platelets in the GCF of patients with periodontitis. These played an antibacterial role in microbiome-triggered periodontitis, independently or interacting with neutrophils. These findings provide a fundamental basis for advancing insight into the inflammatory immune responses associated with periodontitis.

AUTHOR CONTRIBUTIONS

Jie Zhang: Conceptualization, investigation, formal analysis, resources, visualization, and writing—original draft preparation. **Wenjing Li**: Methodology and writing—review and editing. **Hongye Lu**: Methodology and writing—review and editing. **Ruifang Lu**: Methodology, resources, and project administration. **Yalin Zhan**: Conceptualization, methodology, project administration, and writing—review and editing. **Huanxin Meng**: Conceptualization, methodology, project administration, writing—review and editing, and supervision.

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article. This study was supported by research funds from the National Natural Science Foundations of China (NSFC): 81800976 and 81870773.

ETHICS STATEMENT

Ethical approval for this study was obtained from the Ethics Committee of Peking University Health Science Center (PKUSSIRB-201627026) and all subjects provided informed consent prior to their enrolment.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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