Received: 12 August 2019 | Revised: 13 January 2020 | Accepted: 30 January 2020

DOI: 10.1111/jcpe.13265

Journal of Clinical Periodontology WILEY

Platelets as inflammatory mediators in a murine model of periodontitis

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Funding information
This study was supported by research funds from the National Natural Science Foundation of China (NSFC): 81800976; 81870773; 81300879.

Abstract

Aim: To investigate the role of platelets during the development of ligature-induced experimental periodontitis in mice.

Materials and Methods: Experimental periodontitis was induced by placement of sterilized 5-0 cotton ligatures around the maxillary and mandibular second molars of C57BL/6 wild-type mice. Flow cytometry was used to analyse platelet activation and platelet–leucocyte aggregate formation, and histologic analysis was used to evaluate inflammation and localization of platelets and leucocytes in periodontal tissues during the development of experimental periodontitis and in experimental periodontitis with and without antiplatelet drug treatment.

Results: Experimental periodontitis induced platelet activation and platelet–leucocyte interaction. Platelets and leucocytes gradually infiltrated in inflammatory gingival tissues during the development of experimental periodontitis. The inhibition of platelet activation via drug therapy led to significant inhibition of leucocyte migration and marked reduction in periodontal inflammation.

Conclusion: This study revealed that platelets are critical for inflammation and tissue injury in periodontitis and serve as mediators of inflammation in periodontal tissue.

KEYWORDS

host modulation therapy, inflammation, periodontitis, platelet

1 | INTRODUCTION

Platelets are small cell fragments that circulate in the bloodstream and play important roles in inflammation (Li, Yang, Dunn, Gross, & Smyth, 2011; Linden & Jackson, 2010; Shi & Morrell, 2011; Smith & Weyrich, 2011). Platelets participate in tissue injury, immune response and repair, which underlie diverse diseases including autoimmune disorder, atherosclerosis, inflammatory lung and bowel disorder, host defence response and sepsis (Boilard et al., 2010; Kuo, Fan, Dvorina, Chiesa-Vottero, & Baldwin, 2015; Li et al., 2013; Linden & Jackson, 2010; Smith & Weyrich, 2011; Thomas & Storey, 2015; Vowinkel et al., 2007; Zarbock, Singbartl, & Ley, 2006).

Periodontitis is an inflammatory disease caused by host responses against bacterial challenges that leads to the destruction of periodontal tissues (Armitage, 1999). Our previous study showed that platelet function was altered in patients

Zhan and Lu contributed equally to this work.

with periodontitis. High-grade periodontal inflammation was accompanied by a reduction in mean platelet volume, this value increased after active periodontal treatment in patients with severe periodontitis (Wang et al., 2015). Platelets from patients with periodontitis exhibited increased activation compared with platelets from healthy controls (Papapanagiotou et al., 2009; Zhan, Lu, Meng, Wang, & Hou, 2016). Periodontopathogens were shown to be responsible for platelet activation (Assinger, Buchberger, et al., 2011; Assinger, Laky, et al., 2011; Zhan et al., 2016). Periodontal treatment reduces the presence of aggressive, anaerobic species, such as Porphyromonas gingivalis; these species show strong associations with platelet activation (Assinger, Laky, Badrnya, Esfandeyari, & Volf, 2012) and limit platelet activation in patients with periodontitis (Laky et al., 2018). The formation of platelet–leucocyte aggregates, which follows platelet activation, has been suggested as a link between platelets and inflammation (Van Gils, Zwaginga, & Hordijk, 2009). In patients with periodontitis, improvement of the periodontal condition is paralleled by reductions in platelet hyperreactivity and platelet–leucocyte aggregation (Arvanitidis, Bizzarro, Alvarez Rodriguez, Loos, & Nicu, 2017). Circulating leucocytes with attached, activated platelets display a more adhesive phenotype and have an enhanced propensity for phagocytosis (Sreeramkumar et al., 2014). Activated platelets drive responses targeting leucocytes that modulate the host response to infection in inflammatory lung (Zarbock et al., 2006) and bowel disorder (Vowinkel et al., 2007), and sepsis (Clark et al., 2007). Studies from our laboratory have revealed that the numbers of platelet–leucocyte aggregates were significantly elevated in patients with periodontitis compared with controls. Moreover, platelets accumulated within inflamed gingiva in patients with periodontitis, platelets exhibited direct binding to venular endothelium and leucocytes (Zhan et al., 2016, 2017). Although the pathophysiological significance of platelet accumulation in the inflamed gingiva remains poorly understood, there is evidence suggesting that platelets may contribute to periodontal inflammation and amplify the inflammatory response.

In the present study, we investigated the roles of platelets in periodontal inflammation and periodontitis pathogenesis using a murine model of ligature-induced periodontitis.

Clinical Relevance

Scientific rationale for the study: Evidence of platelet migration to infectious tissues suggests that platelets participate in immune inflammatory responses. Neutrophils scan for activated platelets to initiate inflammation. Understanding the role of platelets in the pathogenesis of periodontitis may facilitate new therapeutic approaches for periodontitis.

Principal findings: Platelet activation and platelet–leucocyte aggregate formation were observed with gradual infiltration of platelets and leucocytes in inflammatory gingival tissues during the development of experimental periodontitis. Inhibition of platelet activation led to marked reduction in periodontal inflammation.

Practical implications: Platelets contribute to the pathogenesis of periodontitis. Platelets may represent a novel and effective therapeutic target for host modulation therapy in patients with periodontitis.

2 | MATERIALS AND METHODS

2.1 | Animals

Eighty male C57BL/6 wild-type mice were purchased from Vital River Laboratory Animal Technology (Beijing, China) and used for experiments at the age of 8 weeks. Experiments were performed in accordance with relevant institutional guidelines for the care and use of laboratory animals. The study protocol was approved by the Animal Welfare Ethics of Peking University Biomedical Ethics Committee (LA2018132).

2.2 | Ligature-induced experimental periodontitis

The mice were anaesthetised via intraperitoneal injection. Experimental periodontitis was induced by gentle placement of sterilised 5-0 cotton ligatures around the bilateral maxillary and mandibular second molars, followed by mesiobuccal knotting. Fifty mice were randomly divided into ligature group and control group, and each group was further randomly divided into five subgroups based on the duration of the experiment: 1, 3, 5, 7 and 10 days. In mice with ligature-induced experimental periodontitis, the ligatures were maintained in position throughout the duration of the experiment (1, 3, 5, 7 or 10 days) without any need for replacement. Mice were sacrificed at 1, 3, 5, 7 and 10 days, respectively. Negative controls were sham-ligatured mice, which were sacrificed at corresponding time points.

2.3 | Flow cytometric analysis of platelet activation and platelet–leucocyte aggregate formation in mice with experimental periodontitis

To measure in vivo platelet reactivity, blood collected from mice was rapidly measured for surface expression of P-selectin (CD62P) and CD40L on platelets, and for formation of platelet–leucocyte/neutrophil aggregates using whole-blood flow cytometry.

CD41-PE, CD62P-Alexa Fluor 647, CD40L-PerCP-Cy5.5, CD45-FITC, CD11b-BV605, Ly-6G-PerCP-Cy™5.5 and their isotype controls were obtained from Becton Dickinson Immunocytometry Systems. Blood samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson). The forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain. Platelets were identified

on their characteristic light scattering on FSC/SSC dot-plot and the expression of CD41 (Figure S1). Leucocytes were identified on characteristic light scattering on FSC/SSC dot-plot and expression of CD45 (Figure S2). The neutrophil gate was characterized by high side scatter and CD45 expression and confirmed by characteristic expression of CD11b and Ly-6G. The thresholds for positive cells were set with the appropriate isotype controls (Figures S1 and S2). The percentages of positive cells were calculated.

Exposure of platelet activation markers (CD62P and CD40L) was determined within the platelets gate. Of the gated leucocytes and neutrophils, the extent of binding of anti-CD41 was determined, reflecting the formations of platelet–leucocyte aggregates and platelet–neutrophil aggregates.

2.4 | Histology and immunohistochemistry of periodontal tissues

Mice were sacrificed at the predetermined time points. Their jaws were fixed in 4% paraformaldehyde at 4°C for 24 hr and then decalcified in 10% EDTA (PH 7.2 ± 0.2) at 4°C for 2 weeks (with ×3 solution changes per week). Serial paraffin sections of 5-μm thickness were obtained from the buccal-lingual aspects of the second molars and stained with haematoxylin and eosin (H&E) for histological/histometric evaluation. The locations of platelets in periodontal tissues were detected by immunocytochemistry using a rabbit monoclonal antibody to platelet-specific marker CD41 (Abcam) in accordance with the manufacturer's instructions.

2.5 | Drug intervention

The other 30 mice were randomly divided into ligature-induced experimental periodontitis group and control group, and each group was further randomly divided into three subgroups based on the treatments as follows: vehicle (NaCl)-treated, "NaCl"; aspirin-treated, "Asp"; clopidogrel-treated, "Clop"; periodontitis (P) + vehicle-treated, "P+NaCl"; periodontitis + aspirin-treated "P+Asp"; and periodontitis + clopidogrel-treated, "P+Clop." Mice in three periodontitis groups were subjected to ligature-induced experimental periodontitis as described earlier in this paper (ligatures were maintained for 10 days). One day after placement of ligatures, animals were administered daily doses of intragastric aspirin (Asp; 30 mg/kg), clopidogrel (Clop; 75 mg/kg) or the same volume of vehicle (NaCl 0.9%), this treatment was performed for the remaining 9 days of the study. These doses were previously shown to cause effective inhibition of platelet aggregation and thrombus formation in mice (Sasaki, Ishii, Giddings, & Yamamoto, 1996; Taka, Okano, Seiki, & Yamamoto, 1999). Mice in the other three groups were negative controls with no ligature-induced experimental periodontitis; these mice were treated with Asp, Clop and NaCl 0.9%, as described above. The mice were sacrificed at 10 days after ligature placement.

2.6 | Blood cell analysis

A technician who was blinded to the animal groupings performed blood cell analysis of blood samples using a calibrated Sysmex XS-1000 automated haematology analyser (Sysmex).

2.7 | Evaluation of platelet activation and platelet–leucocyte aggregate formation in mice with antiplatelet drug treatment

Flow cytometric analyses of platelet activation and platelet–leucocyte aggregate formation were identical to the methods described above.

2.8 | Histology and immunohistochemistry of periodontal tissues

Histologic and immunohistochemical analyses were identical to the methods described above.

2.9 | Histomorphometric analysis

The severity of inflammation in connective tissue between the highest peak of alveolar bone crest (ABC) and the cementoenamel junction (CEJ) was classified using inflammation scoring, as described previously (Liu et al., 2006). Severity was ranked as follows: 0, normal tissue (<5% inflammatory cells); 1, mild inflammation (5%–20% inflammatory cells); 2, moderate inflammation (20%–50% inflammatory cells); and 3, severe inflammation with a mass of inflammatory cell infiltration (>50% inflammatory cells). Bone loss was measured as the distance between ABC and CEJ. Five randomly chosen sections of five mice per group were measured. The blinded analysis was conducted by a masked and calibrated examiner using an optical microscope (BX51/DP72; Olympus).

2.10 | Statistical analysis

All statistical analyses were performed using SPSS software, version 19.0 (SPSS). The normality of the data distribution was assessed using the Kolmogorov–Smirnov test. Data sets did not exhibit normal distributions were log-transformed before statistical analysis. A one-way ANOVA followed by Bonferroni correction for post hoc multiple comparisons were used for the comparisons among different experimental time points and treatments in both ligature-induced experimental periodontitis and control groups. The comparisons between ligature-induced experimental periodontitis group and control group for each experimental time point and treatment were performed using Student's *t* test. In addition, Kruskal–Wallis one-way ANOVA and the Mann–Whitney *U* test

were used to evaluate inflammatory intensity. Data are presented as means ± standard deviations (*SD*). Two-tailed *p*-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Experimental periodontitis induced platelet activation and platelet–leucocyte interaction

Flow cytometric analyses of whole blood at 1, 3, 5, 7 and 10 days after ligature placement revealed platelet activation and platelet–leucocyte aggregate formation as indicated by significant increases in the respective percentages of CD62P- and CD40L-positive platelets (Figure 1a,b), and percentages of leucocytes and neutrophils positive for the platelet-specific marker CD41 (Figure 1c,d). These percentages increased as the time of ligature induction increased (Figure 1e).

3.2 | Platelet aggregates in inflammatory gingival tissues of mice with experimental periodontitis

Histological analysis of periodontal tissues following ligature application revealed infiltration of inflammatory cells with platelet aggregates in gingival tissue, which increased as the time of ligature induction increased (Figure 2). The progression of periodontitis increased with an increasing number of platelet aggregates.

The gingival connective tissues in no-ligature control mice were composed of densely packed, organized and interlacing collagen bundles. There was no significant infiltration of inflammatory cells (Figure 2a1). The histopathology of initial stage within 1 day of the

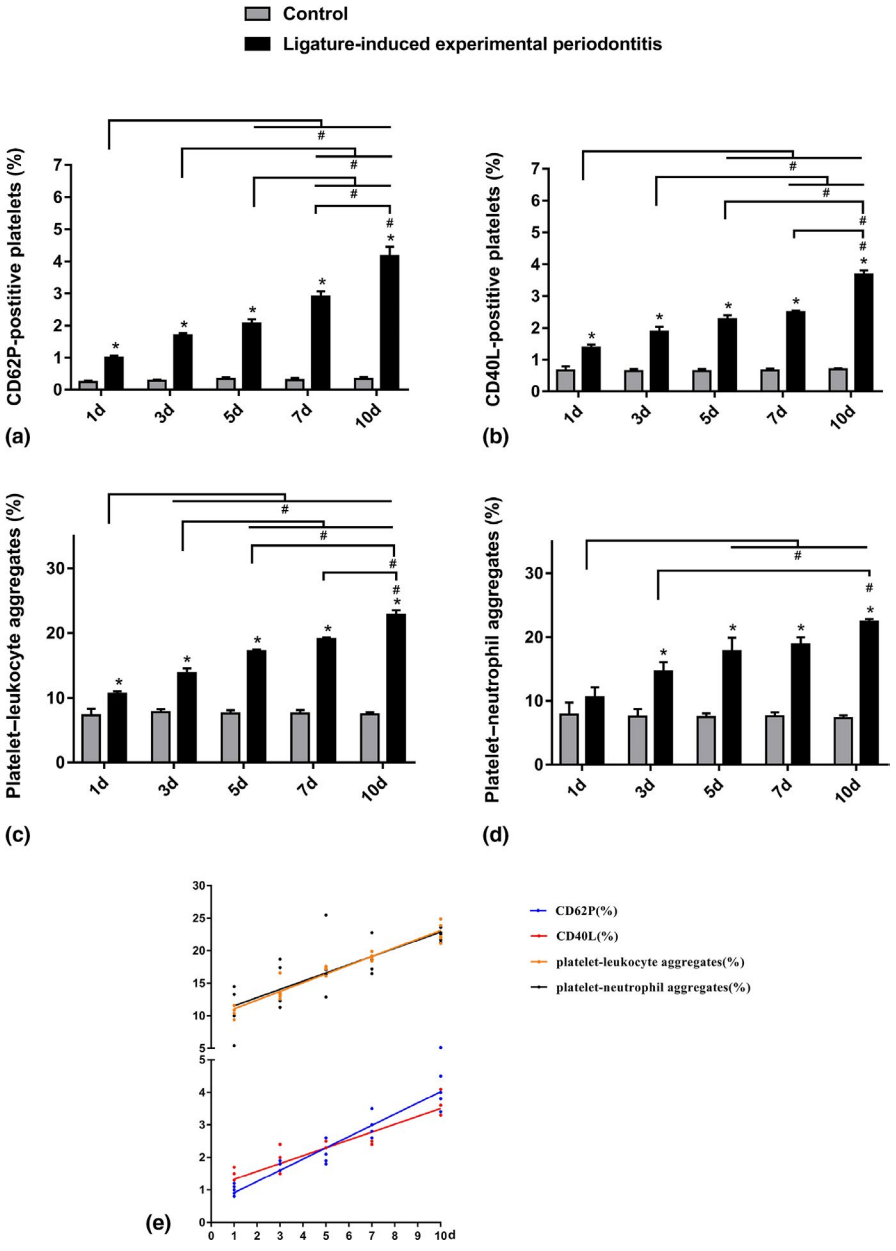

FIGURE 1 Platelet activation and platelet–leucocyte aggregate formation in mice with experimental periodontitis. Percentages of (a) CD62P- and (b) CD40L-positive platelets, (c) platelet–leucocyte aggregates and (d) platelet–neutrophil aggregates increased in mice with ligature-induced experimental periodontitis compared with controls. Data are presented as means ± *SD*. These percentages increased as the time of ligature induction increased (e). Overall *p*-values of the comparisons among different time points within the mice with ligature-induced experimental periodontitis were <.001 (one-way ANOVA); $^{\#}p < .05$ after Bonferroni correction in post hoc testing. Comparisons between mice with ligature-induced experimental periodontitis and control mice for each time point were analysed by Student's *t* test (*$p < .05$, ligature-induced experimental periodontitis vs. control)

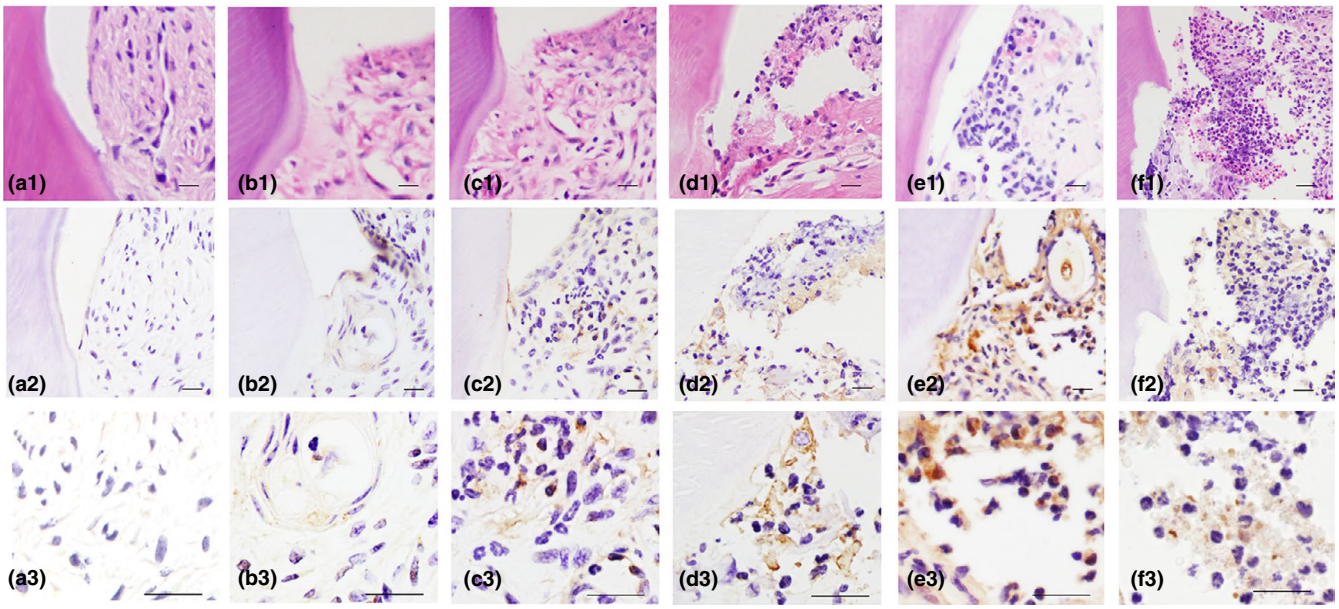

FIGURE 2 Platelet localization in inflammatory gingival tissues of mice with experimental periodontitis. (a1–3) Histological images of minimal inflammatory cells and platelets in control mice. (b1–3) After 1 day of ligature induction, infiltration of a few inflammatory cells and loss of minimal alveolar bone, combined with the presence of platelet aggregates, were observed in periodontal tissue. (c1,2,3–f1,2,3) As the time of ligature induction increased, inflammatory cell infiltration and platelet aggregates increased. Scale bars, 50 μm (a1,2–f1,2) and 20 μm (a3–f3)

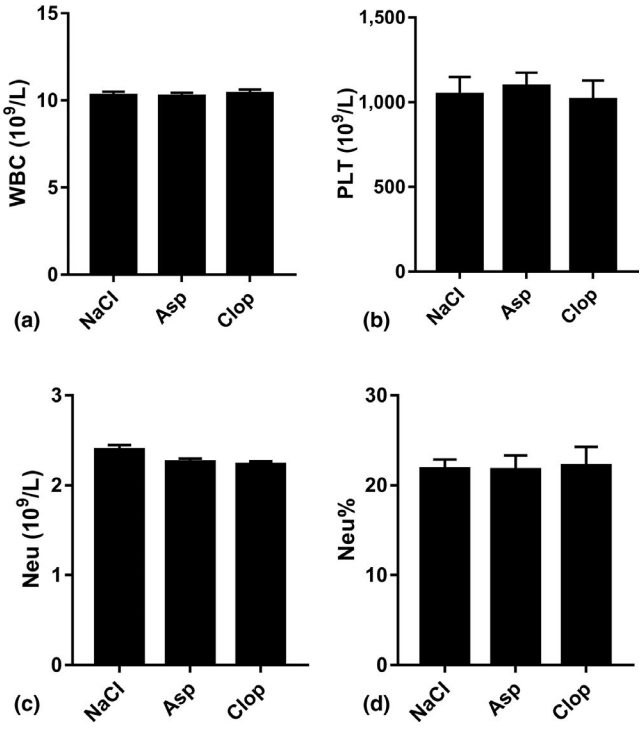

FIGURE 3 Effects of Asp and Clop on circulating blood cells. (a) white blood cell, (b) platelet, (c) and neutrophil granulocyte counts and (d) the percentage of neutrophil granulocytes were not significantly affected by treatment with either Asp or Clop. Data are presented as means ± *SD*

accumulation of plaque at the site revealed elevated vascular vasodilation and migration of relatively small amounts of leucocytes (primarily neutrophils; Figure 2b1), with few platelets (Figure 2b2,b3) throughout the gingival connective tissue across the junctional epithelium. The gingival of early stage after 3 days of continued plaque accumulation showed capillary proliferation, increased transmigration of leucocytes, degeneration of fibroblasts and destruction of collagen, which resulted in collagen-depleted connective tissue (Figure 2c1). Aggregates of platelets were observed in the gingiva; these platelets were colocalized with and attached to leucocytes (Figure 2c2,c3). In the established stage, after 5 or 7 days of continued plaque accumulation, there was significant infiltration of inflammatory cells, which occupied a considerable volume of inflamed connective tissues. Large numbers of infiltrating cells could be identified around blood vessels, adjacent and lateral to the junctional and sulcular epithelia, and between collagen fibre bundles, where these cells caused further tissue destruction (Figure 2d1,e1). Platelet aggregation and adherence to leucocytes was also found in areas of inflammatory cell infiltration (Figure 2d2,d3,e2,e3). Advanced lesion formed after 10 days of continued plaque accumulation. The connective tissue contained dilated capillaries, dense inflammatory cell infiltration, and collagen breakdown (Figure 2f1). The epithelial connective tissue interface and large areas of collagen-depleted connective tissue showed infiltration of numerous inflammatory cells and platelets, along with intercellular oedema (Figure 2f2,f3).

3.3 | No changes were observed in circulating blood cells after Asp or Clop treatment

Mice without ligature-induced periodontitis did not exhibit significant hemodynamic changes after intragastric administration of Asp or Clop (Figure 3).

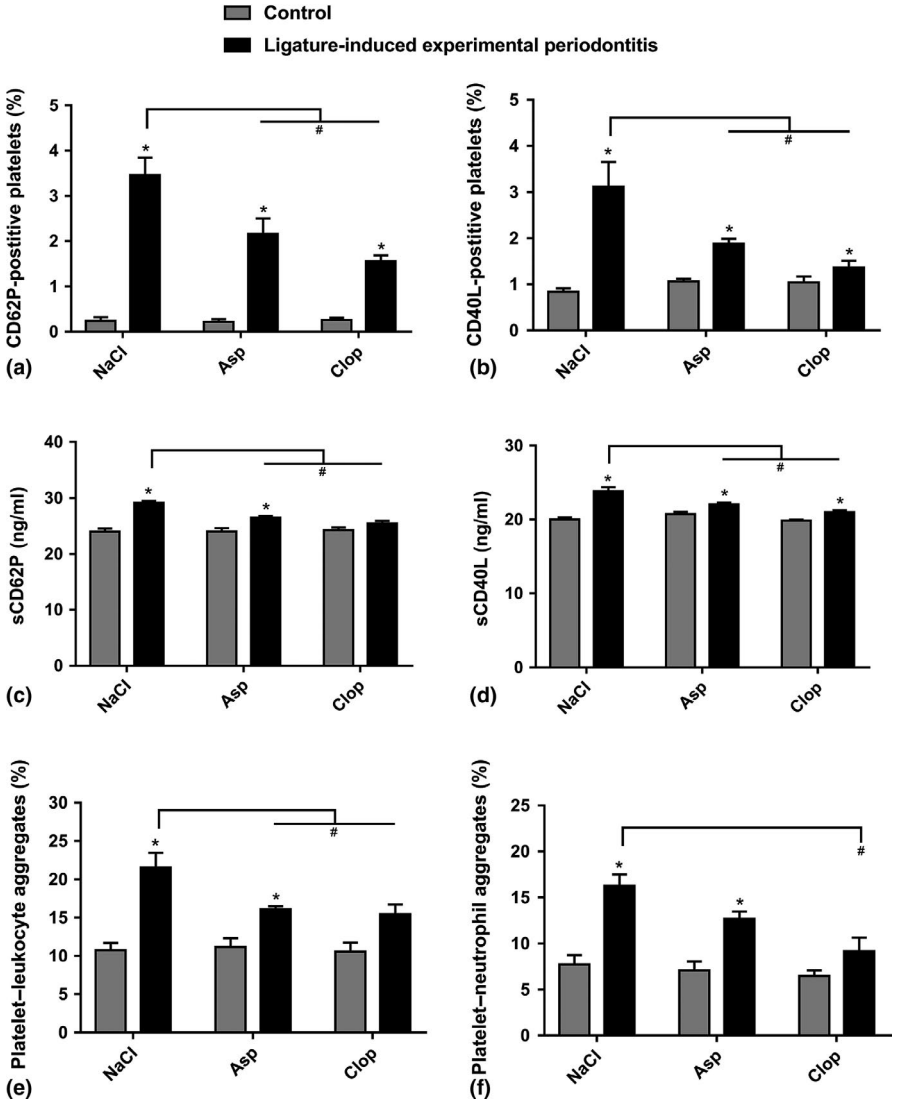

FIGURE 4 Effects of treatment with Asp or Clop on platelet activation, platelet–leucocyte aggregate formation and platelet–neutrophil aggregate formation in mice with ligature-induced experimental periodontitis. Both Asp and Clop inhibited platelet activation, reduced the percentages of platelets positive for expression of (a) CD62P, and (b) CD40L in whole blood and reduced the concentrations of (c) sCD62P and (d) sCD40L in plasma of mice with ligature-induced experimental periodontitis, respectively. Both Asp and Clop reduced the percentages of (e) platelet–leucocyte aggregates and (f) platelet–neutrophil aggregates in whole blood of mice with ligature-induced experimental periodontitis. Data are presented as means ± *SD*. Treatment with Asp or Clop reduced platelet activation, platelet–leucocyte aggregate formation, and platelet–neutrophil aggregate formation in mice with ligature-induced experimental periodontitis; data were analysed by one-way ANOVA, and overall *p*-values of the different treatments within the mice with ligature-induced experimental periodontitis were <.05; $^{\#}p < .05$ after Bonferroni correction in post hoc testing. Comparisons between mice with ligature-induced experimental periodontitis and control mice for each treatment were analysed by Student's *t* test ($^{*}p < .05$, ligature-induced experimental periodontitis vs. control)

3.4 | Effect of inhibition of platelet activation on platelet–leucocyte interaction

In mice with ligature-induced experimental periodontitis, systemic administration of Asp or Clop reduced platelet activity compared to that in saline-treated mice (Figure 4a–d). Consistent with the reduction in platelet activity, significant reductions in the formation of platelet–leucocyte aggregates and platelet–neutrophil aggregates were observed in mice with ligature-induced experimental periodontitis treated with Asp or Clop (Figure 4e,f).

3.5 | Effect of inhibition of platelet activation on periodontal tissue inflammation

Regardless of treatment with NaCl, Asp or Clop, the periodontal tissues of mice from non-ligatured groups exhibited healthy gingiva without alveolar bone resorption (Figure 5a–c). Ligature placement for 10 days induced an inflammatory response and osteoclastic resorption of alveolar bone, similar to that in human periodontitis (Figure 5d). There were significant increases in the numbers of adherent platelets and leucocytes that infiltrated connective tissues

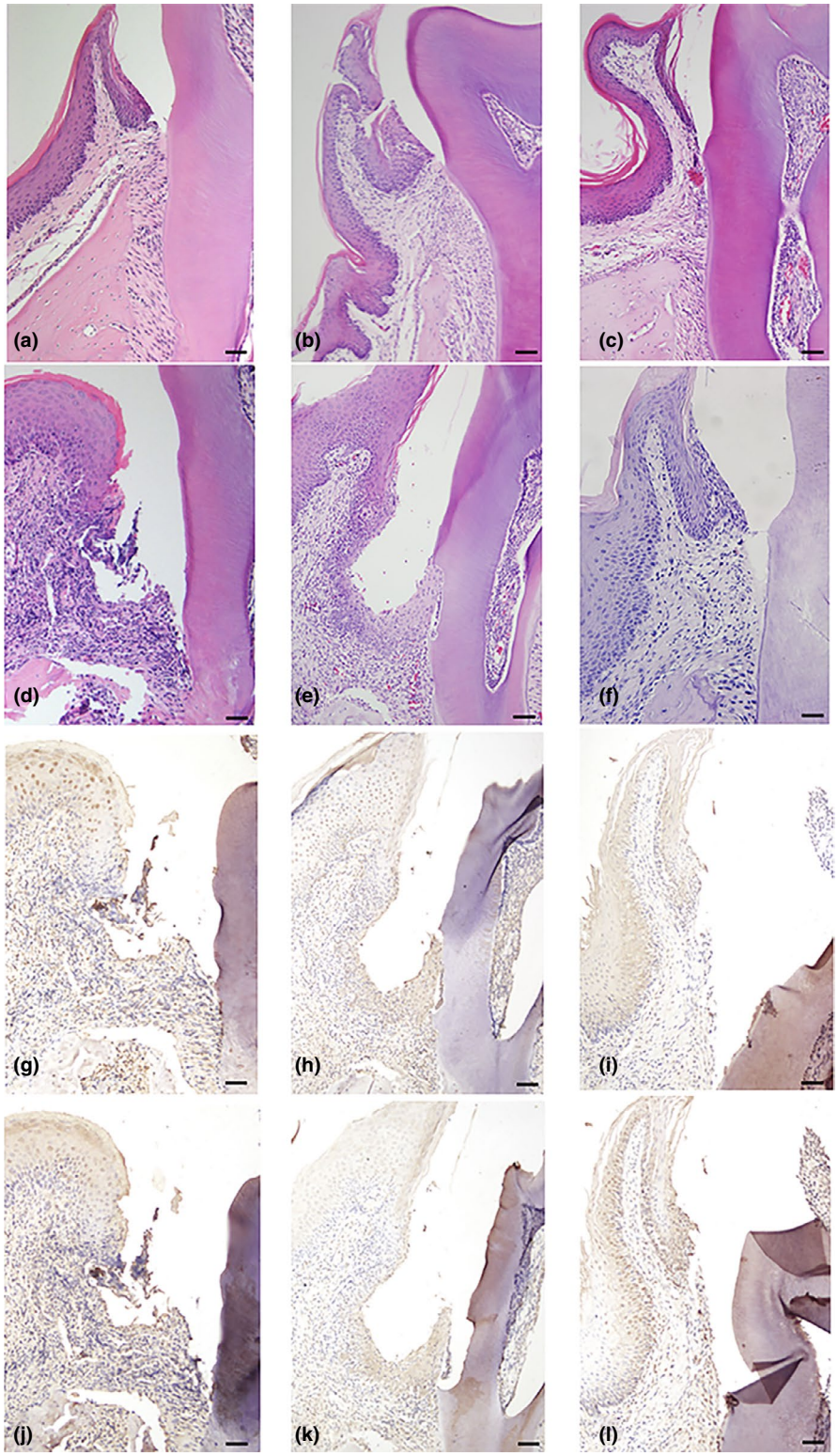

FIGURE 5 Histological and immunohistochemical appearances of periodontal tissue in mice treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) following control or ligature-induced experimental periodontitis modelling. Healthy periodontal tissue was collected from control mice without ligature-induced experimental periodontitis, which were treated with NaCl (a), Asp (b) or Clop (c). In mice with experimental periodontitis, the presence of tissue destruction was associated with bone resorption and intense inflammatory infiltration (d), these were reduced by treatment with Asp (e) or Clop (f). Immunohistology staining for CD41 and CD45 showed that platelets and leucocytes were distributed on inflamed gingival tissue of mice treated with NaCl 0.9% following ligature-induced experimental periodontitis modelling (g and j). Few (h and k) or almost no (i and l) platelet and leucocyte were observed on gingival connective tissue of mice treated with Asp (h and k) or Clop (i and l) following ligature-induced experimental periodontitis modelling. Images are representative of the results observed in 5 mice in each group. Scale bars, 50 μm

(Figure 5g, j). The distribution of the platelets was consistent with the extent of leucocytes infiltration (Figure 5g, j). Asp or Clop treatment significantly reduced the infiltration of adherent platelets (Figure 5h, i) and leucocytes (Figure 5k, l). In addition, administration of Asp or Clop during the periods of experimental periodontitis led to reductions in periodontal inflammation (Figure 5e,f). Asp-treated mice with ligature-induced periodontal disease exhibited reductions in inflammatory cell infiltrate and increased amounts of collagen fibres (Figures 5e and 6a). Alleviation of the inflammatory response and tissue damage during experimental periodontitis was significantly more effective with Clop treatment than with Asp treatment (Figure 6a). Histologic examination of Clop-treated mice revealed reduced vascular vasodilation, mild collagen destruction, slight bone resorption and minimal apical migration of junctional epithelium (Figure 5f).

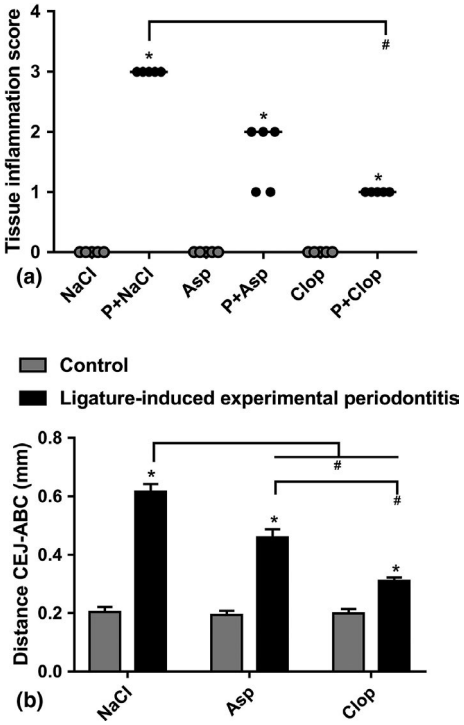

FIGURE 6 Effects of treatment with Asp or Clop on severity of inflammation and bone loss following ligature-induced experimental periodontitis modelling. Five haematoxylin-and-eosin-stained slides per mouse taken from five mice in each group with or without ligatures that were treated with NaCl 0.9%, Asp (30 mg/kg), or Clop (75 mg/kg) were analysed for the intensity of inflammatory infiltrate (a) (Data are presented as median). Data were analysed by Kruskal–Wallis one-way ANOVA and the overall *p*-values of comparisons among treatments within the experimental periodontitis groups are <.05, $^{\#}p < .05$ in multiple comparison testing; $^{*}p$-values of comparisons between ligature-induced experimental periodontitis and control mice are <.05 for each treatment by the Mann–Whitney *U* test. And the distance between CEJ and alveolar bone crest (b) (Data are presented as means ± *SD*). Data were analysed by one-way ANOVA, and the overall *p*-values of comparisons among treatments within the experimental periodontitis groups are <.001, $^{\#}p < .05$ after Bonferroni correction in post hoc testing; $^{*}p$-values of comparisons between ligature-induced experimental periodontitis and control mice are <.05 for each treatment by Student's *t* test by an experienced pathologist who was blinded to the experimental grouping data

Histologic examination revealed collagen destruction and alveolar bone loss in mice with ligature-induced experimental periodontitis (Figure 5d). The reduced CEJ–ABC distance in mice with Asp or Clop treatment indicated decreased bone resorption by systemic administration of antiplatelet activity drugs, compared with vehicle-treated mice; this effect was more prominent in Clop-treated mice (Figure 6b).

4 | DISCUSSION

The function of circulating platelets is altered in patients with periodontitis. Our studies of human periodontitis have revealed the colocalization of platelets and leucocytes in inflamed periodontal tissue (Zhan et al., 2017). These cell–cell interactions have been described in several inflammatory diseases (Weyrich & Zimmerman, 2004). The present study showed that platelet activation played a crucial role in the development of periodontitis, particularly during early stage of periodontal inflammation. The inhibition of platelet activation and disruption of platelet–leucocyte interactions led to reduced leucocytes migration and permeability, as well as reduced periodontal inflammation in mice with experimental periodontitis. This study revealed that platelets are critical for inflammation and tissue injury in periodontitis and serve as mediators of inflammation in periodontal tissue.

Ligature-induced experimental periodontitis resulted in platelet activation and platelet–leucocyte interaction, consistent with previous research involving patients with periodontitis (Papapanagiotou et al., 2009; Zhan et al., 2016, 2017). Both human and animal studies have identified an association between periodontitis and increased platelet activation compared with healthy controls. Here, the state of platelet activation was assessed during the development of ligature-induced periodontitis. Platelet activation and platelet–leucocyte aggregate formation increased as the time of ligature induction increased, revealing a positive association between platelet activation and time of ligature induction. Periodontal inflammation progressed with increasing platelet activation.

The progression of periodontitis in mice exhibits characteristics similar to those of human disease. Inflammatory cell and platelet aggregate infiltration increased with modelling time. In early stage, aggregates of platelets were observed in gingiva; these platelets were colocalized with and attached to leucocytes. In the established stage of disease, platelets aggregation and adherence to leucocytes could be identified around blood vessels and between collagen fibre bundles. Advanced lesions showed infiltration with numerous inflammatory cells and platelets, along with intercellular oedema. Thus, in mice with ligature-induced experimental periodontitis, platelets participated in the early development of periodontitis.

Leucocytes are primary effectors of immune responses against invading pathogens, as well as central mediators of inflammatory injury. Both functions rely on the remarkable ability to migrate within and through blood vessels. In mice with ligature-induced experimental periodontitis, we found that inflammatory cells migrated within and through blood vessels to reach periodontal tissue. An association between platelets and leucocytes in terms of migration to inflamed periodontal tissue was manifested by the appearance of platelet–leucocyte aggregates in systemic venous blood and by platelets' binding to adherent leucocytes in gingiva and venules of periodontal tissue. It has been suggested that neutrophils scan for activated platelets to initiate inflammation; aggregates may represent an important source of inflammatory mediators that can sustain or amplify inflammatory responses (Sreeramkumar et al., 2014). However, the pathophysiological significance of platelet–leucocyte aggregates in periodontitis remains poorly understood.

Immune inflammatory responses require leucocytes to roll, adhere and transmigrate through vascular endothelium into inflammatory

tissue and thus participate in host defence. These processes can be promoted by the formation of platelet–leucocyte aggregates (Köhler et al., 2011; Zarbock et al., 2006). As platelets become activated, they express CD62P on their surfaces (Holmes, Sobel, Howard, & Schneider, 1999). Membrane-expressed CD62P engages its receptor PSGL-1, a critical step in the activation and recruitment of leucocytes to inflammatory sites (Phillipson & Kubes, 2011; Ridley et al., 2003). Selectin-mediated platelet-neutrophil interactions are a critical step in the activation and recruitment of leucocytes to lung in acute lung injury (Zarbock et al., 2006). As described above, activated platelets drive responses targeting leucocytes that modulate the host response to infection. Our previous study provided evidence that patients with periodontitis had increased platelet activation compared with healthy controls (Zhan et al., 2016). In present study, platelet activation in mice with ligature-induced experimental periodontitis increased as the time of ligature induction increased. As platelets become activated, platelet–leucocyte aggregates were formed (Holmeset al., 1999; Phillipson & Kubes, 2011; Ridleyet al., 2003). The parallel increase and consistent distributions of platelets and leucocytes in inflamed gingival tissue suggest the possibility of interplay between platelets and leucocytes in the response to periodontal infection.

In accordance with this relevant biologic role of platelets, platelet–leucocyte aggregates were found in periodontal tissue during periodontitis. Antiplatelet drugs may affect periodontitis pathogenesis through their inhibitory effects on platelet and leucocyte interactions. Previous studies showed that antiplatelet drugs reduced the immunoinflammatory response in a rat model of periodontal disease (Coimbra, Steffens, Muscarães, Rossa, & Spolidorio, 2014). In present study, we evaluated the effect of antiplatelet drugs on numbers of circulating leucocytes and platelets. The dosing regimen did not induce thrombocytopenia or leucopenia that could compromise the results, consistent with the findings of a recently published study (Gupta & Eisen, 2009).

Systemic administration of antiplatelet drugs with the same dose range established in the literatures was effective for reducing both platelet activation and platelet–leucocyte aggregate formation (Ma et al., 2001; Sugidachi, Asai, Ogawa, Inoue, & Koike, 2000; Wallace, Soldato, Cirino, & Muscara, 1999) during the induction of periodontitis. A significant reduction in the influx of inflammatory cells was observed in gingival tissue, along with reduced alveolar bone loss. A novel finding of this study was that the migration of leucocytes in inflamed periodontal tissue was profoundly reduced upon inhibition of platelet activation, suggesting that platelet activation plays a major role in the inflammatory response and tissue injury during experimental periodontitis. The results of this study are consistent with the findings of a study of experimental colitis, which showed that the recruitment of leucocytes and the recruitment of platelets in inflamed colonic venules were co-dependent processes (Vowinkel et al., 2007). In addition, the binding of platelets to leucocytes in periodontal venules may result in enhanced leucocyte activation, which increases the avidity and/or expression of adhesion molecules on the leucocytes (Suzuki et al., 2001). Notably, a recent study highlighted the role of platelet–neutrophil interactions in promoting formation of neutrophil extracellular trap (NET; Clark et al., 2007). NETs are a potential source of proteases that could contribute to vascular injury during periodontitis; their presence may explain how inhibition of platelet activation abrogates the increase in vascular permeability in experimental periodontitis. However, it remains unclear whether NETs are generated in the inflamed periodontal microvasculature. Additional studies are needed to provide a better understanding of the molecular mechanisms modulating the interactions of platelets and leucocytes in the periodontal microenvironment.

These observations also point to the importance of platelets in tissue injury and repair, where platelets may participate in vascular damage and angiogenesis (Anderson et al., 2001; Golino et al., 1997; Ross, 1999; Weber, Zucker, & Schror, 1999). Recent studies revealed that systemic administration of antiplatelet drugs attenuated the inflammation associated with periodontitis in rats without influencing the repair process upon stimulus removal (Coimbra et al., 2011). Platelets are an excellent source of mitogens, matrix metalloproteinases (Falcinelli, Ciferri, & Gresele, 2001) and lysosomal enzymes (Ciferri et al., 2000), which influence resident structural cells and the composition of extracellular matrix (ECM) in the vasculature. Thus, the role of activated platelets in lesion development may be attributed to P-selectin contact-dependent delivery of platelet-derived mitogens to tissue (Burger & Wagner, 2003; Weber et al., 1999). Thrombi are known to release platelet-derived growth factor, epidermal growth factor, insulin-like growth factor and transforming growth factor beta, thereby providing a matrix for growth of smooth muscle cells (Ip et al., 1991). Previous studies suggested a critical role for platelet adhesion in the initiation of atherosclerotic lesion formation. The inhibition of platelet adhesion to the vascular endothelium profoundly attenuated these processes (Fingerle, Johnson, Clowes, Majesky, & Reidy, 1989). Our results emphasize that tissue remodelling events are dependent on platelet activation.

In summary, this study derived novel evidence that platelets may contribute to periodontitis pathogenesis. Our results show that platelets are dynamic participants in the multicomponent system responsible for periodontal inflammation and injury; the interdependent processes of the recruitment of leucocytes and platelets in inflamed periodontal tissue depend upon platelet activation. Our findings, coupled with previously published observations, indicate that platelets may represent a novel and effective therapeutic target for modulation of the inflammatory cell infiltration associated with periodontitis.

ACKNOWLEDGEMENTS

We especially thank Yi Song, a statistician at the Institute of Child and Adolescent Health, School of Public Health, Peking University, with her help for statistical analysis.

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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SUPPORTING INFORMATION

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How to cite this article: Zhan Y, Lu R, Meng H, et al. Platelets as inflammatory mediators in a murine model of periodontitis. *J Clin Periodontol*. 2020;47:572–582. https://doi.org/10.1111/jcpe.13265