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Original Article

Neutrophil extracellular traps induced by diabetes aggravate periodontitis by inhibiting janus kinase/signal transducers and activators of transcription signaling in macrophages

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Abstract *Background/purpose:* Diabetes, which is a systemic disease, increases susceptibility to destructive periodontal diseases, which are characterized by infectious susceptibility, but the potential mechanisms remain unknown. The aim of this study was to investigate the mechanism of high glucose environment promoting the occurrence and development of local periodontal inflammation.
Materials and methods: In this study, the effects of neutrophil extracellular traps (NETs) on macrophage polarization and the mechanism were designed to verify whether this course plays a role in periodontal tissue impairment associated with diabetes. Here, we examined the impact of NETs on macrophages in vitro. NETs were isolated from cultures of neutrophils exposed to hyperglycemia. Mouse models of diabetic periodontitis (DP) and macrophage polarization were developed, and the degrees of NET formation in the periodontal tissue of DP mice were assessed. Furthermore, western blotting was performed to analyze the related mechanisms.
Results: The results revealed that hyperglycemia induced the formation of NETs, and abundant NET formation led to proinflammatory cytokine secretion by macrophages and low expression of JAK-2 and STAT-3 in vitro and in vivo. NETs regulated macrophage polarization through the JAK/STAT pathway.
Conclusion: These results suggest that NETs target proinflammatory cytokine secretion via the JAK/STAT pathway and may play important roles in DP progression and macrophage polarization, which indicates that therapeutically referring to this regulatory pathway might be a promising method for treating diabetes-associated inflammatory diseases.

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Introduction

As it known to all, diabetes mellitus (DM), which is featured by insulin resistance, hyperglycemia, and phlogosis disorders, is one of the fastest growing serious chronic diseases in the 21st century.[1,2] Periodontitis (PD) has global prevalence, making it the sixth most common disease in the world.[3] Thus, DM and PD have a bidirectional relationship, and PD is a complication of DM.[4] DM, especially uncontrolled DM, leads to an increase in the prevalence of PD.[5]

Diabetic periodontitis (DP) is showed by severe damage of the periodontium, which indicates coordinated activities at the molecular level. In healthy adults, neutrophils are the most content type of blood cells, contributing to 50.0 %—70.0 % of circulating blood cells, and are the most common cells associated with innate immune responses.[6] Neutrophils release neutrophil extracellular traps (NETs) after being stimulated by irritants. While studies have indicated that NETs are related to the manage of infectious pathogens, recent studies have shown that the external environment, such as hyperglycemia, can also induce NETs.[7,8] A NET is a network structure with DNA as the framework that is inlaid with proteins such as histone (H), myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G (CG), and protease 3 (PR3).[9]

Mucosal inflammation around the teeth and the spoil of underlying bone are common features of PD in humans.[10] Many studies have highlighted that NETs are abundant in the periodontal pocket/gingival crevice and inflamed periodontal tissues.[11—13] Notably, evidence shows that deleting NET significantly reduces alveolar bone loss in vivo.[14] Therefore, exploring the underlying mechanism by which NETs regulate inflammatory homeostasis might have pivotal clinical value for achieving healthy periodontal maintaining in DP patients. However, the mechanism remains unclear.

Macrophages are not only the main cellular component of innate immunity, but also play a significant role in tissue homeostasis.[15,16] Macrophages differentiate into different functional phenotypes, such as M1 macrophages (typical maker was iNOS) and M2 macrophages (typical maker was ARG-1), to cope with different external environments.[15,17]

Here, we hypothesized that a high glucose environment activated neutrophils to produce NETs and that the existence of large quantities of NETs could affect the polarization of macrophages, such as anti-inflammatory M2 macrophages. We studied the mechanisms by which NETs regulate macrophage polarization in high glucose environments. Our work highlights NETs as a critical bridge between DM and PD and gain a deeper understanding of the role of neutrophil activation and tissue immunity.

Materials and methods

Animals and ethics approval

Male wild-type C57Bl/6J mice aged 6～8 weeks were purchased from Tong Xiang Branch of Zhejiang Vital River Laboratory Animal Co. Ltd. The animals were allowed to freely eat and drink under reverse conditions of a 12:12 h light-dark cycle, a controlled temperature between 21 and 23 °C, and controlled humidity between 40% and 70%. All animal experiments were performed in accordance with the Animal Care and Use Committees and under NIH guidelines for proper animal welfare.

Neutrophil isolation and activation

Isolated neutrophils from male adult wild-type C57Bl/6J mice (aged 6～8 weeks) by using a Mouse Bone Marrow Neutrophil Isolation Kit (Solarbio, Beijing, BJ, China) according to the instructions. Cells were cleaned and resuspended in RPMI 1640 medium (Gibco, New York, NY, USA). Neutrophils (5×10^5 cells/well) were added to 12-well plates. In separate experiments, cells were presented under the condition of 0, 5.5 and 25 mM glucose at 37 °C for 6 h. After stimulation of neutrophils, cells were washed three times with PBS and fixed with 4 % paraformaldehyde.

To isolate NET, fresh RPMI were added in each well and NETs adherent to the plate were collected after vigorous agitation. The medium was centrifuged at 20×*g* for 5 min and supernatant phase, containing NETs, was collected and stored at −20 °C until use.

Quantification and image analysis of NETs

NET DNA generated by neutrophils was digested with 500 mU/ml micrococcal nuclease (MNase). A PicoGreen dsDNA Assay Kit (Fushen Bio, Shanghai, SH, China) was selected to investigate dsDNA referring to the manufacturer's protocol. As previously described, the supernatant was removed and replaced with PBS, and added an equal amount of 1X Quant-iT PicoGreen reagent, incubated for 5 min at room temperature.[18] Fluorescence was detected with a spectrofluorometer (Tecan, Männedorf, Zurich Switzerland) at an wavelength of 488 nm and 525 nm.

Neutrophils were incubated with specific primary antibodies against Ly6G (1:100, Proteintech, Chicago, IL, USA) and MPO (1:100, Proteintech). Scan the slide under the same magnification, exposure time, light intensity, and camera gain conditions.

Cell culture and polarization

RAW 264.7 cells were purchased from Stem Cell Bank, Chinese Academy of Sciences. The cells were incubated at 37 °C under a humidified atmosphere of 5 % CO_2. RAW 264.7 cells were seeded at a density of 1×10^6 cells per well in 6-well plates and then treated with IFN-γ (20 ng/mL, BioLegend, San Diego, CA, USA), LPS (1 μg/ml, InvivoGen, Paris, France), or IL-4 (10 μg/mL, BioLegend) for further experiments.

NET-stimulated macrophages

NETs were collected from 25 mM glucose medium and added to M1(IFN-γ containing medium) and M2 (IL-4 containing medium)medium in an incubator at 37 °C under a humidified atmosphere of 5 % CO_2 for 12 h.

Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants was collected from NET-stimulated macrophages and centrifuged at 12,000 rpm at 4 °C for 10 min, and the concentrations of TNF-α, IL-1β and IL-10 were measured with ELISA kits (Boster, Wuhan, Hu Bei, China). All samples were measured at a wavelength of 450 nm.

Animals and treatment

All experimental protocols were approved by the Ethical Committee of Animal Care and Experimental Committee of the Hunan University of Tradition Chinese Medicine (LLBH-202311280001).

Streptozotocin (STZ, Sigma–Aldrich, Darmstadt, Germany) at a dose of 200 mg/kg was intraperitoneally injected into the mice as previously described.[19] After one week of accommodation, the mice received STZ treatment. Prior to STZ administration, food was removed from the animal cages for over 16 h. Water was provided as normal. The experimental scheme is shown in Fig. 3. The animals were randomly divided into 4 groups (n = 8): (1) the unligated group (Ctl), (2) the ligated group (PD), (3) the STZ-injected group (DM), and (4) the STZ-injected + ligated group (DM + PD). To induce PD in mice, a 5–0 silk ligature (Johnson & Johnson, New Jersey, USA) was tied around the second maxillary molar.[20] The ligations remained intact throughout the experimental period. After 21 days, the mice were euthanized, and the maxillae were removed for further analysis.

Micro–computed tomography (micro-CT)

To evaluate alveolar bone loss, the maxillae were dissected to quantitatively analyze the alveolar bone. Then, the hemimaxillae were examined by a micro-CT system (μCT 50, Scanco Medical AG, Bassersdorf, Switzerland) as previously described.[21]

Hematoxylin and eosin and immunofluorescence staining

Hematoxylin and eosin (HE) and immunofluorescence staining were performed as previously described.[22] For immunofluorescence staining, all antibody kits were used according to the instructions. NETs (MPO+), M1 macrophages (iNOS+), and M2 macrophages (CD206+) were examined using ImageJ.

Western blot analysis

Cells and tissues were washed twice with PBS and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein samples were separated on polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated with the appropriate primary antibodies: GAPDH (1:5000, BBI, Shanghai, China), β-actin (1:2000; Servicebio, Wuhan, Hu Bei, China), Arg-1 (1:1000; Servicebio), JAK-2 (1:1000; Servicebio), phospho-JAK-2 (pJKA-2) (1:1000; Servicebio), STAT-3 (1:1000; Affinity, London, UK) and phospho-STAT-3 (pSTAT-3) (1:1000; Abclonal, Wuhan, Hu Bei, China).

Statistical analysis

The results are presented as the mean ± standard deviation (SD). Statistical analysis using ANOVA and Student's t test. A value of $P < 0.05$ was considered statistically significant.

Results

High glucose induces neutrophils to form extracellular traps

To investigate the effect of hyperglycemic conditions on NET formation, we separated neutrophils from healthy mice under the condition of different concentrations of glucose. The normal group showed uniform cell staining and intact capsules. As shown in Fig. 1, in the low glucose group, some cells showed extracellular extensions and were stained positive, indicating the formation of NET structures. The cells in the high glucose group showed many extracellular extension structures and were stained positive, hinting the formation of NETs. Next, the concentration of NETs was measured by examining cell-free dsDNA. The results showed in Fig. 2A that the amount of NET formation was higher than that in the 25 mM group ($P < 0.01$).

NETs regulate macrophage polarization

To explore the impact of NETs generated under high glucose conditions on macrophage polarization, two different phenotypes of macrophages were cocultured with NETs. The data showed that the overproduction of proinflammatory cytokines was associated with the presence of glucose-induced NETs. The ELISA results showed that IL-10

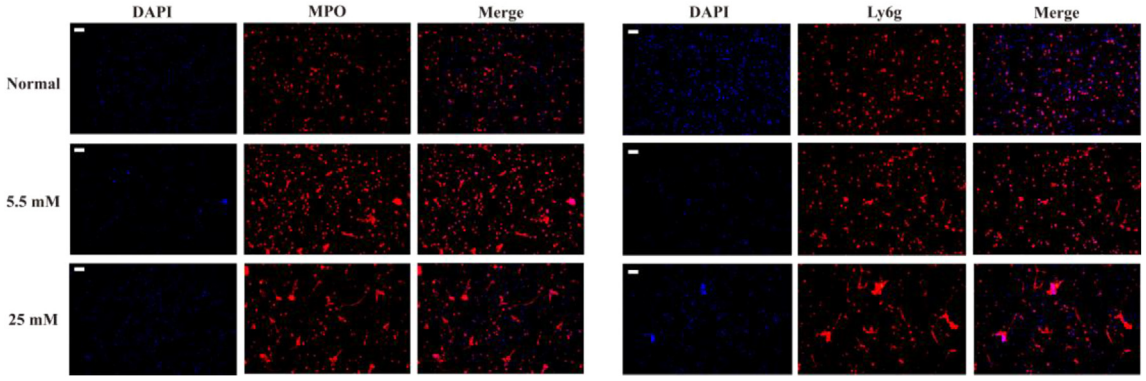

Figure 1 The normal group had uniform cell staining and intact capsules. In the 5.5 mM group, some cells showed extracellular extension structures and were stained positive, indicating the formation of NET structures. The cells in the 25 mM group showed many extracellular extension structures and were stained positive, indicating the formation of NETs. The amount of NET formation was higher than that in the low-glucose group (scale bar 100 μm).

production declined when NETs were present, while TNF-α and IL-1β levels rapidly increased (Fig. 3). These results showed significant activation of inflammatory macrophages during high glucose stimulation.

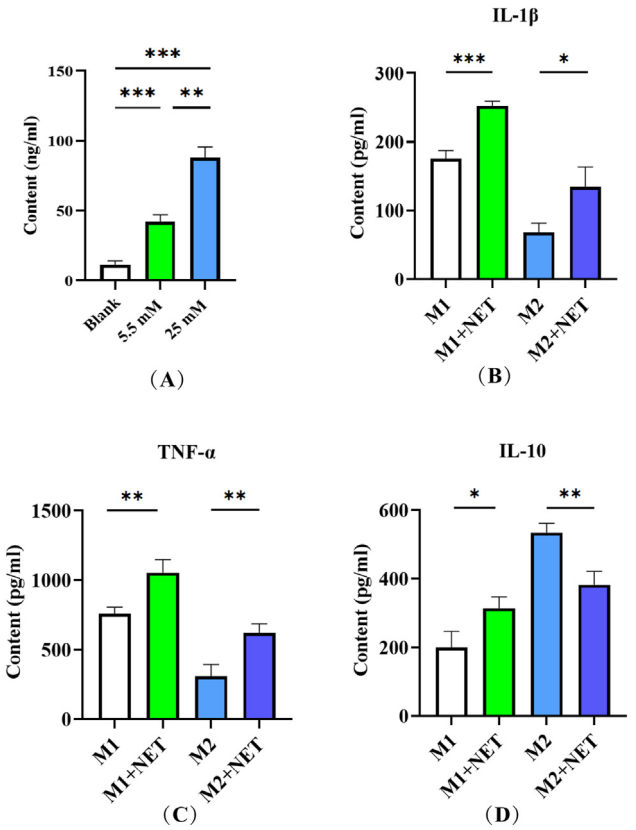

Figure 2 NETs regulate macrophage polarization: (A) picogreen dsDNA assays. As glucose increased, the levels of NETs gradually increased, and there was a significant difference between the groups. (B ~ D) The ELISA results showed that NETs could promote macrophage secretion of the proinflammatory factors IL-1β and TNF-α while inhibiting the secretion of the anti-inflammatory factor IL-10 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

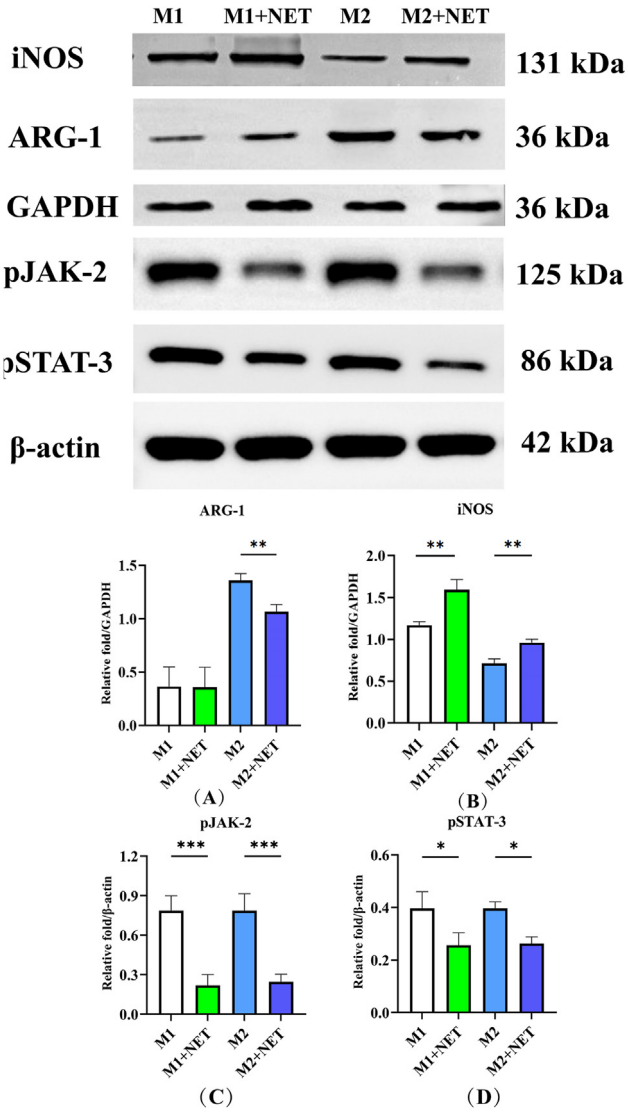

Figure 3 (A ~ D) ARG-1, iNOS, p-JAK-2 and p-STAT-3 protein expression in macrophages cocultured with NETs (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

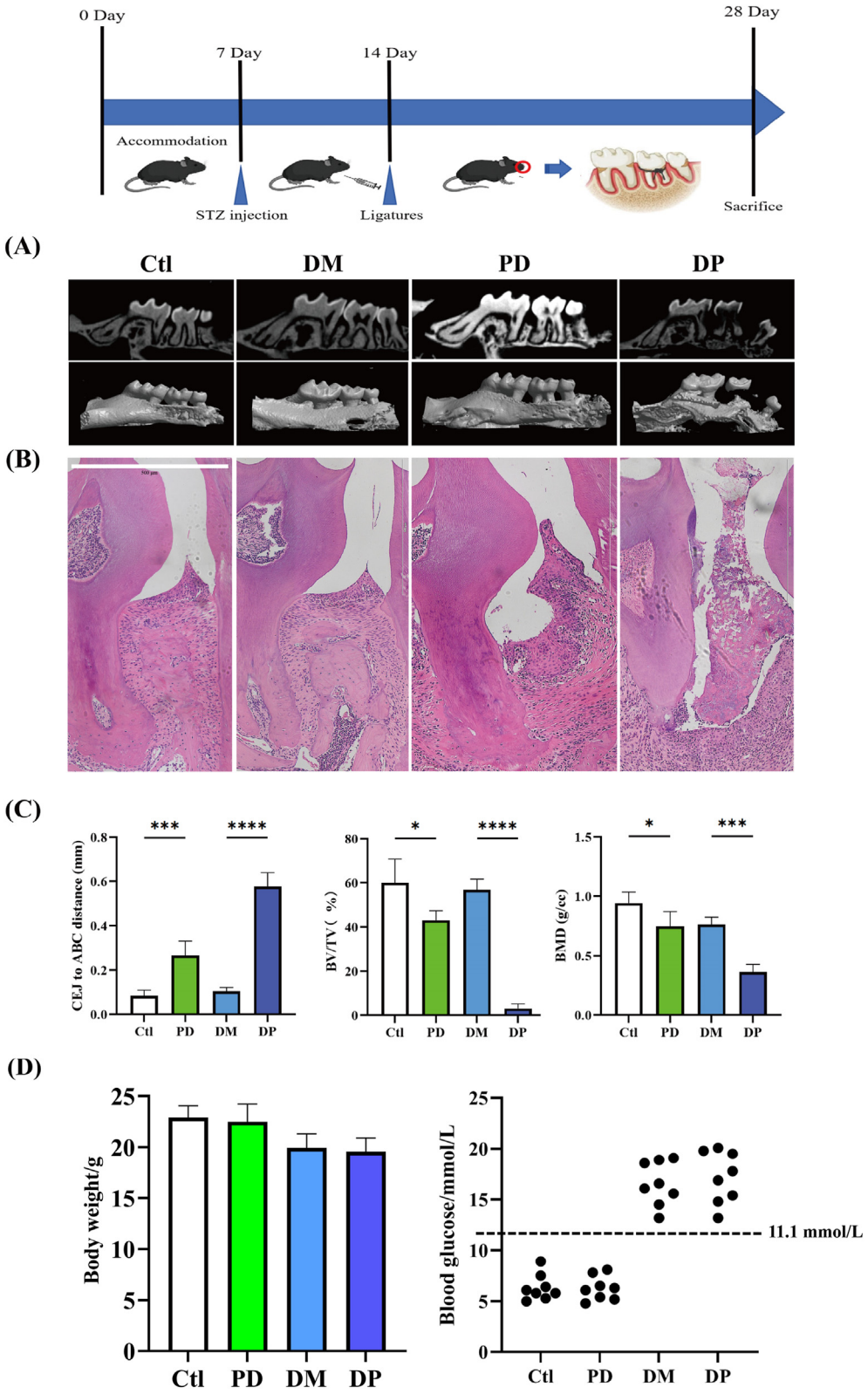

Figure 4 NETs regulate macrophage polarization in vivo: (A) 3D reconstruction of maxillary molars on the palatal side. (B) Histological observations of the changes in bone volume, as shown by H&E staining. (C) Micro-CT analysis of alveolar bone loss. (D) After 7 days of STZ injection, mice fasting glucose after immunization and body weight (*$P < 0.05$, ***$P < 0$ .001, ****$P < 0.0001$, scale bar 500 μm).

NETs induce macrophage polarization via the JAK-STAT pathway

To further explore the mechanism of NETs regulating macrophage polarization, the impact on the JAK-STAT pathway was further evaluated. Exposure of macrophages to NETs reduced the expression of pJAK-2 and STAT-3, which are markers of the JAK-STAT pathway and are related with macrophage polarization. Thus, inhibition of the JAK-STAT pathway have a connection with proinflammatory activation and reduced anti-inflammatory functions in macrophages.

Hyperglycemia promotes the progression of PD

The classic ligature-induced PD (Lip) mouse model was used to probe the influence of diabetes on PD progression. After injection of STZ, successfully induced diabetes mice (Fig. 4D). Compared with non-STZ mice, the weight of diabetes mice decreased significantly. The ligature process was performed for 21 days in the PD model. Micro-CT and histomorphometric analyses were performed to observe alveolar bone loss and inflammation (Fig. 4A and B). The ligated mice showed marked increases in the distance from the CEJ to the ABC and notable decreases in the BV/TV (Fig. 4C). Many MPO + NETs were detected in the periodontium in the STZ-injected + ligated group (Fig. 5A and B).

NET aggregate leads JAK-STAT pathway is a key player in NET-induced macrophage polarization

To verify the mechanism of JAK-STAT pathway on the progression of diabetes periodontal disease. On the basis of previous in vitro experiments, an in vivo LIP model was conducted. NET content has also been detected. Histone H3 aggregated in PD and DP model. The results are similar to the in vitro results (Fig. 6): p-JAK and p-STAT proteins and NETs exhibit opposite trends. Thus, the expression of inflammatory factors increases with the weakening of the JAK-STAT signaling pathway.

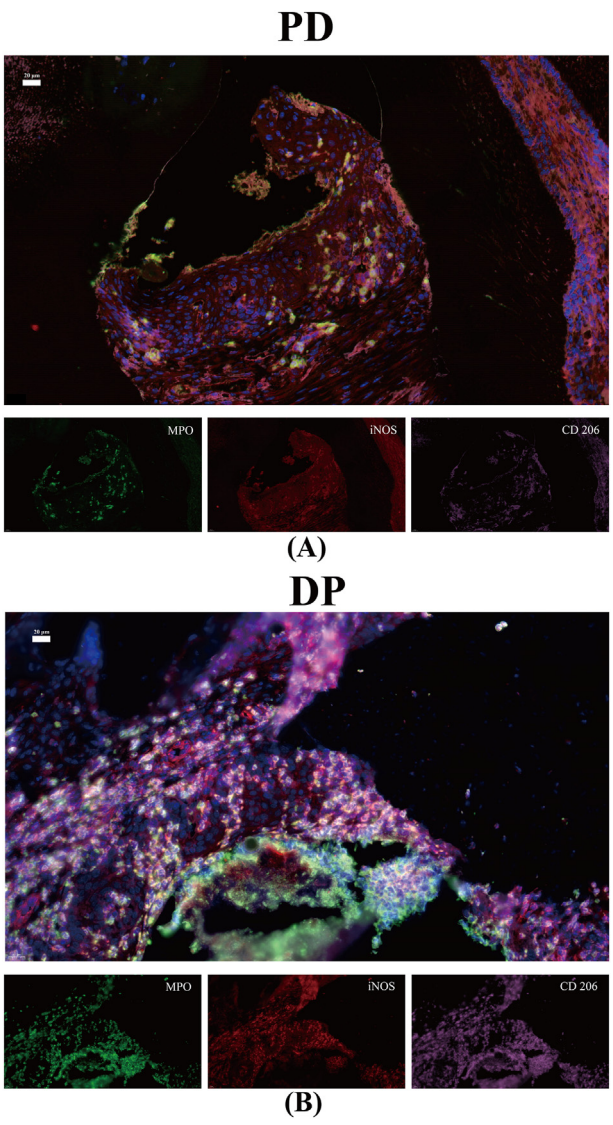

Figure 5 The JAK-STAT pathway is a key player in NET-induced macrophage polarization: (A, B) Immunohistological analysis and analysis of the mRNA expression of MPO, CD 206 and iNOS.

Discussion

NETs are discovered in human and murine PD, and in mice, they are connected with increased inflammatory progression.[13,14] NETs are also observed in diabetic mice.[8] Although NETs are regarded as generally inflammatory, and it is not entirely clear know how NETs promote macrophage activation. In this study, we confirmed that the interaction between NETs and macrophages plays a pivotal role in DP. By placing neutrophils isolated from healthy mice in hyperglycemic environment, large amounts of NETs were formed. In vitro, the presence of NETs could accelerate macrophage polarization toward the proinflammatory M1 phenotype and suppress M2 polarization. We also detected an increase in NETs in DP mice, which was consistent with an in vitro study that verified that diabetes primed neutrophils for NET formation and induced M1 polarization by inhibiting the JAK-STAT pathway.

Neutrophils, as the first line of defense against infection, act a indispensable role in maintaining tissue homeostasis. Emerging evidence has suggested that NETs are the bridge between diabetes-associated diseases.[23–26] For example, NETs stimulate NLRP3 inflammasome activation and glomerular endothelial dysfunction under high glucose conditions in vitro and in vivo. In addition, inhibiting NET formation relieves endothelial disorder and renal damage in diabetic kidney disease.[26]

Macrophages play a fundamental part in maintaining the homeostasis of the tissue microenvironment and in the progression of PD.[15,16] In response to different factors, macrophages exhibit plasticity; they can polarize and form different functional phenotypes.[27] M1 and M2 macrophages represent the destructive and restorative stages of PD, respectively. The polarization transition of M2 macrophages helps accelerate periodontal regeneration; On the contrary, it can cause destructive damage.[28,29]

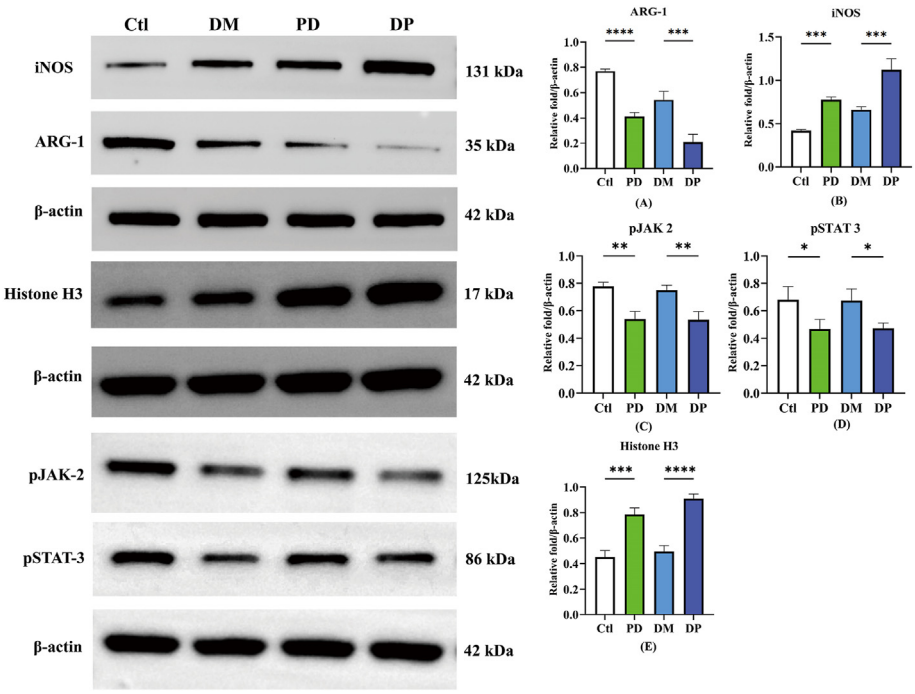

Figure 6 (A～E) ARG-1, iNOS, JAK-2, p-JAK-2, STAT-3 and p-STAT-3 mRNA expression in periodontal tissue (*$P < 0.05$, **$P < 0$ .01, ***$P < 0.001$, ****$P < 0.0001$).

In DP mice, the NET-macrophage interaction is associated with persistent macrophage inflammation. This is supported by our current findings, which showed an increase in inflammatory cytokines and pro-inflammatory macrophages in the second molars of diabetic mice. Furthermore, we provide evidence that NETs are abundant in the lesion. The presence of NETs also increases the secretion of inflammatory cytokines. These data are in line with the relation between NET formation and periodontal unsteadiness.[30]

The molecular networks orchestrating M1—M2 macrophage reprogramming, such as toll-like receptors (TLR)/nuclear factor-κB (NF-κB) and Janus kinase (JAK)/signal transducers and activators of transcription (STAT), are not yet fully understood.[31] We conducted an exploratory study to evaluate the relationship between macrophage phenotypes and NETs. We found that hyperglycemia induced a switch toward M1-polarized macrophages by inhibiting the JAK2/STAT3 pathway. However, whether JAK2/STAT3 pathway activation has a therapeutic effect on alleviating periodontal inflammation needs to be further study.

In summary, our findings disclosed the fundamental part of the JAK-2/STAT-3 pathway in NET-macrophage interactions in the context of diabetes and suggested a strategy to promote inflammation resolution and periodontal repairation by activating JAK-2/STAT-3 pathway. This strategy may be the key to dealing with diabetes and other chronic inflammatory diseases.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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