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Akt2 Affects Periodontal Inflammation via Altering the M1/M2 Ratio

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Abstract

Periodontitis is a bacteria-driven inflammatory destructive disease that leads to attachment loss, bone resorption, and even tooth loss. Accumulating studies revealed that macrophages might play an nonnegligible role during the processes of periodontitis. However, the underlying mechanism remains largely unknown. In this study, we found novel Akt2/JNK1/2/c-Jun and Akt2/miR-155-5p/DET1/c-Jun signaling pathways that regulated the polarization of macrophages and altered periodontal inflammatory status. Through hematoxylin and eosin, immunostaining, and immunofluorescence staining of clinical specimens, a higher number of M1 phenotype macrophage infiltration was found in periodontitis than in normal controls. Flow cytometry and immunofluorescence showed that overexpression of Akt2 in RAW 264.7 cells induced M1 macrophage polarization and decreased M2 polarization, while knockdown of Akt2 exerted an opposite effect. Furthermore, overexpression of Akt2 activated the JNK pathway and then increased the release of proinflammatory mediators, while knockdown of Akt2 downregulated the above genes accordingly. Importantly, the macrophage polarization and the subsequent alteration of pathway molecules induced by overexpression of Akt2 could be rescued by Akt2 and JNK inhibitors. Moreover, JNK inhibition could facilitate M2 polarization of macrophages. In a mouse periodontitis model, the novel signaling pathway as well as clinical phenotype was further verified. Inhibition of Akt2 facilitated macrophage M2 polarization and rescued the bone loss due to periodontitis. Collectively, we identified novel Akt2/JNK1/2/c-Jun and Akt2/miR-155-5p/DET1/c-Jun signaling pathways that regulate macrophage polarization and highlight that Akt2 inhibition promotes M2 polarization of macrophages and can be a novel potential candidate in the treatment of periodontitis.

Keywords: periodontitis, macrophage polarization, JNK signaling pathway, innate immunity, miR-155/DET1, inflammatory environment

Introduction

Periodontitis is a bacterially induced inflammatory disease, which leads to progressive destruction of periodontium (Li et al. 2018; Renn et al. 2018). Moreover, studies have implied associations of periodontitis with systematic diseases, including cardiac diseases, diabetes, rheumatic arthritis, and so on (Michaud et al. 2007; Sun et al. 2017; Chukkapalli et al. 2018). Substantial evidence has revealed that macrophages play vital roles in defending periodontal pathogen infection (Yang et al. 2014). Macrophages exhibit an array of activation status in response to extracellular signals or stimuli, among which are 2 extreme functional outcomes, termed M1 and M2 phenotypes (Cheng et al. 2017; Czimmerer et al. 2018; Yao et al. 2019). M1 phenotype macrophages, polarized under interferon-γ (IFN-γ) and lipopolysaccharide (LPS) stimulation, produce proinflammatory cytokines and mediators such as tumor necrosis factor–α (TNF-α), interleukin (IL)–1β, inducible nitric oxide synthase (iNOS), and so on, leading to tissue inflammation (Gordon and Taylor 2005; Satoh et al. 2010; Sica and Mantovani 2012). However, M2 phenotype macrophages, induced by IL-4 and IL-10, generally express arginase1 (Arg-1) and CD206 at a high level and participate in anti-inflammatory progressions and tissue repair (Varin and Gordon 2009; Satoh et al. 2010; Van Dyken and Locksley 2013). Although accumulating evidence indicates diverse biological functions of distinct macrophage phenotypes, their specific role in the progression of periodontitis remains elusive. Most studies have revealed that M1 phenotype macrophages are predominant in periodontitis (Hussain et al. 2016; Yu et al. 2016, 2018; Zhou

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A supplemental appendix to this article is available online.

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et al. 2019), while some studies uphold enhanced expression of M2 macrophage-associated markers (Gheren et al. 2008; Navarrete et al. 2014). In consideration of the complexity of macrophage polarization in periodontitis, the M1/M2 ratio has been proposed (Yang et al. 2018; Zhou et al. 2019). Some studies have implied that the M1/M2 ratio was significantly higher in patients with chronic periodontitis (Yang et al. 2018; Zhou et al. 2019), but other reports have revealed that the ratio in periodontitis was similar to that in healthy tissues (Garaicoa-Pazmino et al. 2019). Considering that the reparative role of macrophage M2 polarization in autoimmune diseases and inflammatory diseases, it is necessary to investigate whether macrophage M2 polarization can be a novel approach in the treatment of periodontitis and whether induction of macrophage M2 polarization can attenuate the destruction of periodontitis.

Accumulating evidence shows that the PI3K/Akt signaling pathway is sensitive to the changes of microenvironment, and activation of PI3K/Akt signaling is critical for cellular survival, proliferation angiogenesis, and tumorigenesis (Engelman et al. 2006; Li et al. 2015). In the past decades, studies have illuminated the critical role of the activation of the PI3K/Akt signaling pathway in inducing macrophage M2 polarization (Li et al. 2015; Cheng et al. 2017).

However, Akt2, a subset of the Akt family that includes 3 isoforms called Akt1, Akt2, and Akt3 (Cole et al. 2019), has been implied to work adversely in recent years. Studies implied that inhibition of Akt2 might induce macrophage M2 polarization, which is different from traditional views (Kuijl et al. 2007; Androulidaki et al. 2009; Arranz et al. 2012). However, the role of Akt2 in the process of macrophage polarization and its potential mechanism has yet to be illuminated in periodontitis.

Accordingly, this study aimed to clarify roles of Akt2 in the polarization of macrophages and its potential mechanisms in modulating the local inflammatory environment of periodontitis. We hypothesized that selective inhibition of Akt2 could induce macrophage M2 phenotype polarization and reverse locally the proinflammatory microenvironment in periodontitis. To assess this hypothesis, selective knockdown or overexpression of Akt2 has been performed and the phenotype of macrophages and their underlying signaling pathways have been investigated.

Materials and Methods

Gingivae Sample Collection

Gingival tissues were collected from patients seeking dental treatment at Peking University School and the Hospital of Stomatology with informed consent. Our work has been approved by the Biomedical Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB 2016-29 and PKUSSIRB-201951179). For the control group, periodontal probing depth had to be less than 3 mm, with a gingival bleeding index (BI) ≤1, no attachment loss, more than 20 teeth, and no alveolar bone loss on radiographic examination (distance between enamelo-cemental junction and alveolar bone crest less than 2 mm). The control group mainly included patients who received gingivectomy or crown lengthening during orthodontic or prosthodontic treatment. For the periodontitis group, periodontal probing depth had to be more than 6 mm, clinical attachment loss had to be more than 3 mm with a BI ≥2 (Mazza et al. 1981; Garaicoa-Pazmino et al. 2019; Wang et al. 2019; Gao et al. 2020), and obvious bone loss had to be observed on radiographic examination. All patients had to conform to the criteria as follows: 1) no cigarette smoking, 2) no systemic diseases that might influence the periodontal condition, 3) no anti-inflammatory medication intake during the past 3 mo, 4) no periodontal treatment during the past 6 mo, and 5) not pregnant or breastfeeding. The above criteria were formulated according to published work (Garaicoa-Pazmino et al. 2019). Six control and 6 periodontitis biopsies were collected, which included 3 male and 3 female patients, ages 18 to 40 y. After collection, gingival samples were fixed in 4% paraformaldehyde solution for 24 h, which were dehydrated, embedded in paraffin, and sectioned to 5 μm subsequently.

Hematoxylin and Eosin, Immunostaining, and Immunofluorescence Staining

Hematoxylin and eosin (HE), immunostaining, and immunofluorescence staining were processed according to the published work (Zhuang et al. 2019). Briefly, 5 sections from each group were processed. For immunostaining, CD68 (dilution 1:200; Affinity BioScience), CD86 (dilution 1:80; Affinity BioScience), and CD163 (dilution 1:400; Bioss) were selected to detect M1 and M2 polarized macrophages. IL-1β (dilution 1:100; Abcam) and IL-10 (1:100; Santa Cruz) were used to observe the inflammatory status. For immunofluorescence staining, CD68 (Santa Cruz), phosphorylated (*p*)–Akt2 (Bioss), phosphorylated (*p*)–JNK (Affinity BioScience), CD86 (dilution 1:80; Affinity BioScience), and CD163 (dilution 1:400; Bioss) were applied. Details are illustrated in the Appendix.

Cell Culture, Transfection, Infection, and Polarization of Macrophages

A macrophage cell line RAW 264.7 was used to investigate the effects of Akt2/JNK on macrophage polarization in this study. Details about cell culture, transfection, infection, and polarization are described in the Appendix.

Flow Cytometry, Quantitative Real-Time Polymerase Chain Reaction, and Western Blot

Flow cytometry, immunofluorescence, quantitative real-time polymerase chain reaction (qRT-PCR), and Western blot were processed as described previously (Wu et al. 2018; Lu et al. 2019; Zhuang et al. 2019). Details are illustrated in the Appendix. The sequences of target-specific primers are listed in Appendix Table 1.

Murine Ligature-Induced Periodontitis Model and Treatment

Animal experiments were examined by the Biomedical Ethics Committee of Peking University (No. LA2019092). Male C57BL/6 mice aged 6 wk were randomly divided into 3 groups with 5 mice in each group. After general anesthesia, sterile silk ligatures (5-0) were placed at the cervical areas of mice maxillary second molars. The silk ligatures were removed immediately in the control group, while ligatures were kept in site for a week in the experimental periodontitis group and intervention group. During the experiment, the intervention group was injected with Akt2 inhibitor CCT128930 (100 μM, 5 μL) locally in the periodontal region daily. The injection began on the day that the ligatures were installed. The control group was injected with normal saline with the same volume. At 7 d postoperation, mice were euthanized via overdose injection of sodium pentobarbital. Maxilla were removed carefully with surrounding tissues and fixed in 4% paraformaldehyde solution for 24 h for micro–computed tomography (CT) analysis, HE, immunostaining, and immunofluorescence staining.

Statistical Analysis

All numerical data are expressed as mean ± standard deviation. Experiments were repeated at least 3 times. Statistical differences between groups were compared using 1-way analysis of variance (ANOVA) and Tukey's test (SPSS 13.0; SPSS, Inc.). Differences were considered statistically significant at $P < 0.05$.

Results

M1/M2 Phenotype Macrophage Infiltration in Gingival Tissues of Periodontitis

HE staining revealed that, compared with healthy gingivae, significantly higher amounts of inflammatory cells infiltration could be observed in gingival tissues with periodontitis (Fig. 1A). Immunohistochemistry revealed higher expression of IL-1β in periodontitis, while the difference in IL-10 expression between periodontitis and control was not significant (Fig. 1A). Little macrophage infiltration ($CD68^+$) could be observed in healthy gingival tissues, but the numbers of $CD68^+$ cells were significantly higher in the periodontitis group (Fig. 1B, C). The infiltration of both M1 polarized macrophages ($CD86^+$) and M2 polarized macrophages ($CD163^+$) in periodontitis was significantly higher than that in the healthy group (Fig. 1B, C). Colocalization of $CD68^+$ and $CD86^+$ cells in the periodontitis group could be observed, while the amounts of colocalized $CD68^+$ and $CD163^+$ cells were less than CD86/CD68-positive cells (Fig. 1C). The numbers of $CD86^+$ cells and $CD163^+$ cells in the periodontitis group were 60.250 ± 13.036 and 20.375 ± 4.357, respectively ($P < 0.05$; Fig. 1D). The M1/M2 ratio ($CD86^+/CD163^+$) of the periodontitis group was also calculated to assess which phenotype was more predominant in periodontitis, and the ratio was about 2.957. The above data indicated that the M1 phenotype macrophage might be predominant in periodontitis. Immunofluorescence costaining exhibited higher *p*-Akt2 and *p*-JNK expression in infiltrated macrophages ($CD68^+$) of periodontitis (Fig. 1E, F), which implied that phosphorylated Akt2 and JNK in infiltrated macrophages could play vital roles in periodontitis.

Akt2 Inhibition Facilitates M2 Phenotype Polarization of Macrophages

Akt2 was knocked down via specific small interfering RNA (siRNA), and its efficiency and specificity have been examined ($P < 0.05$; Appendix Fig. 1 and Appendix Fig. 2). Flow cytometry and immunofluorescence revealed that LPS stimulation significantly increased the percentage of M1 polarized macrophages. However, Akt2 inhibition attenuated the effects of LPS. The LPS-induced M2 phenotype polarization was not significant, while the M2 phenotype macrophages were significantly increased after Akt2 inhibition (Fig. 2A, B). qRT-PCR revealed that LPS stimulation could markedly increase the expression of M1 phenotype macrophage-associated genes, including IL-1β, IL-6, iNOS, and TNF-α, while selective knockdown of Akt2 could decrease their transcription ($P < 0.05$; Fig. 2C). In addition, Akt2 inhibition could rescue the reduction of Arg-1 induced by LPS (Fig. 2D). After M2 polarization via IL-4 and IL-13 stimulation, flow cytometry and immunofluorescence revealed a higher percentage of M2 phenotype macrophages, which were further augmented after Akt2 inhibition. The percentage of M1 phenotype macrophages also decreased after Akt2 inhibition (Fig. 2E, F). Moreover, qRT-PCR and Western blot revealed significantly higher expression of Arg-1 via IL-4 and IL-13 stimulation, which was further increased after Akt2 inhibition ($P < 0.05$; Fig. 2G, H). Collectively, the above data implied that inhibition of Akt2 could facilitate M2 phenotype polarization of macrophages.

Overexpression of Akt2 Attenuates M2 Phenotype Polarization of Macrophages

Akt2 was overexpressed via a lentivirus-carrying *Akt2* expression cassette. Successful transfection and its specificity were checked via immunofluorescence, qRT-PCR, and Western blot ($P < 0.05$; Appendix Fig. 3 and Appendix Fig. 4). Flow cytometry and immunofluorescence of cells exhibited higher amounts of M1 macrophages after LPS stimulation. Akt2 overexpression further increased the percentage of M1 macrophages and decreased M2 macrophages (Figs. 3A and 4B). Moreover, the transcription levels of IL-1β, IL-6, iNOS, and TNF-α were also augmented after Akt2 overexpression ($P < 0.05$; Fig. 3C). The expression of Arg-1 was attenuated via LPS stimulation, which was further decreased after Akt2 overexpression (Fig. 3D). After M2 polarization, higher percentages of M2 phenotype macrophages were observed by flow cytometry and immunofluorescence assays, and Akt2 overexpression could reduce the percentage of M2 phenotype macrophages. In

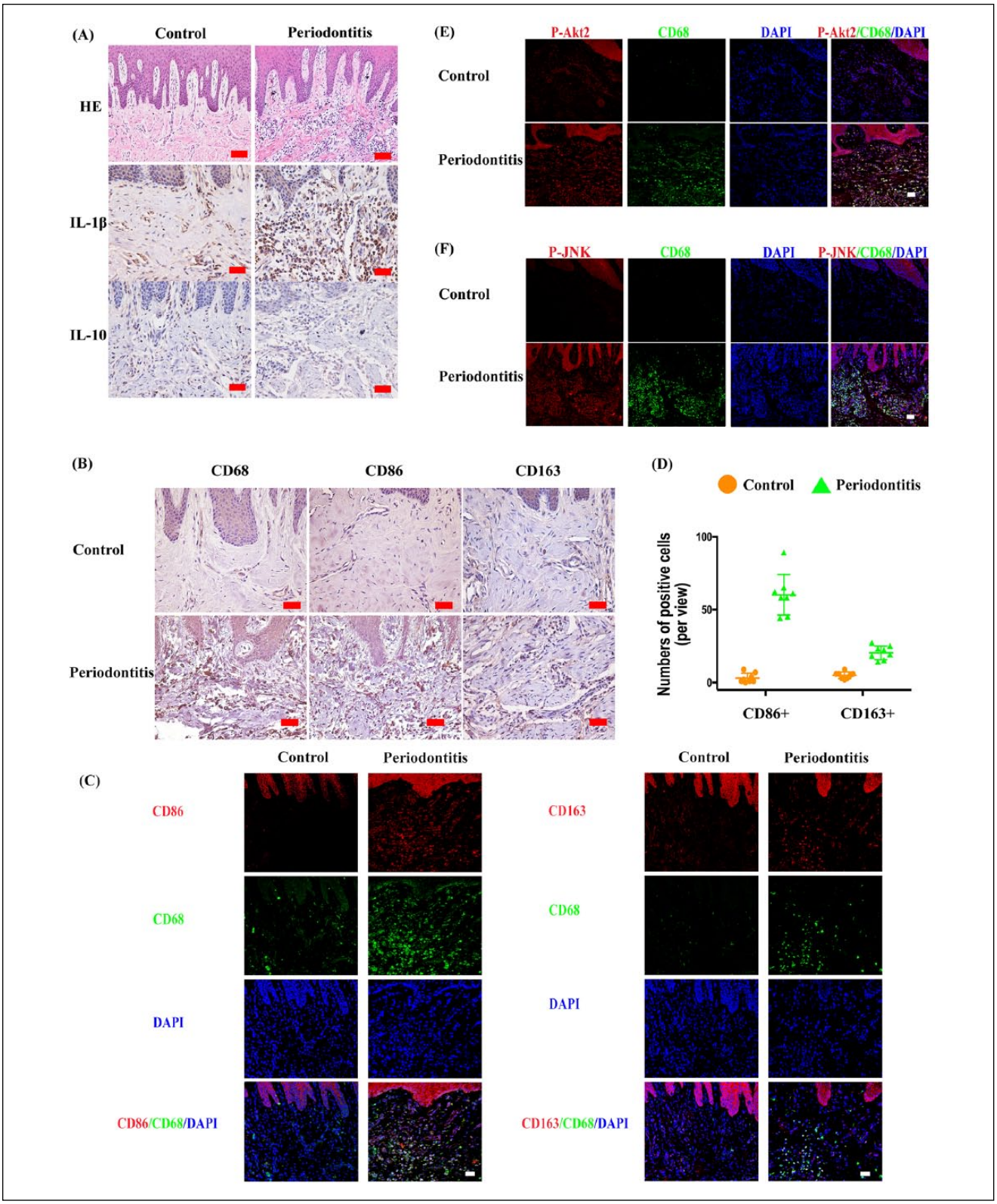

Figure 1. The filtration of macrophage in the buccal surface of gingivae of periodontitis and its polarization. (**A**) Hematoxylin and eosin staining (scale bar = 200 μm) and immunohistochemistry staining (scale bar = 100 μm) of interleukin (IL)–1β and IL-10 in the gingivae of healthy individuals and those with periodontitis. (**B**) Immunostaining of gingival tissues, in which CD68 represents macrophage, CD86 represents M1 phenotype macrophage, and CD163 represents M2 phenotype macrophage. Scale bar = 100 μm. (**C**) Representative images showing colocalization of CD86 (M1) and CD163 (M2) positive cells with infiltrated macrophage ($CD68^+$) in healthy and periodontitis gingivae (scale bar = 50 μm). (**D**) The number of M1 and M2 phenotype macrophages at 400× magnification of healthy individuals and those with periodontitis based on immunostaining results of CD68, CD86, and CD163. (**E**, **F**) Representative images showing *p*-Akt2 and *p*-JNK expression in infiltrated macrophages in healthy and periodontitis gingivae (scale bar = 50 μm).

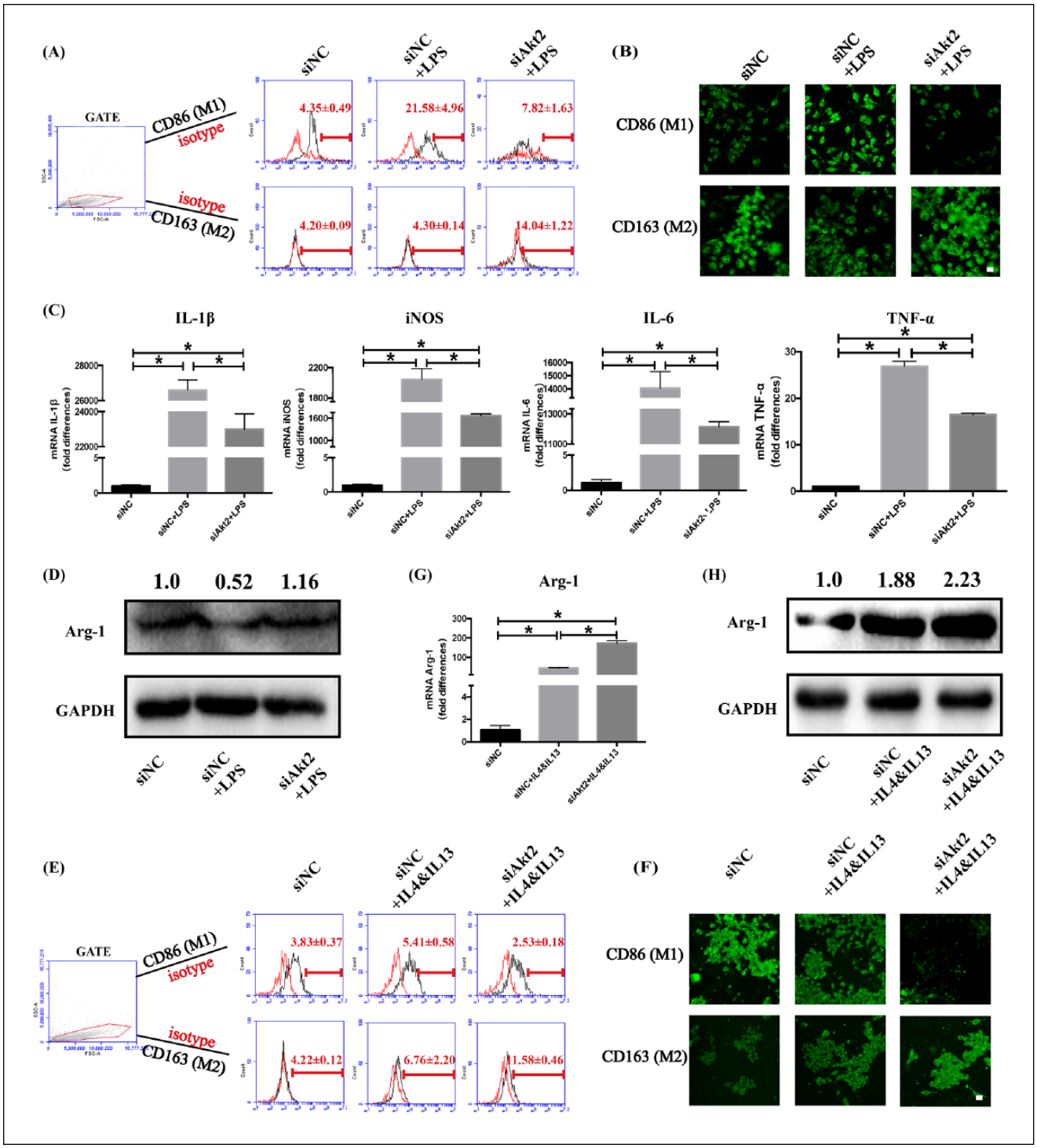

Figure 2. Knockdown of Akt2 promotes M2 polarization of macrophages. Cells were transfected with siNC and siAkt2, respectively, and exposed to lipopolysaccharide (LPS) for 12 h. (**A**) CD86-APC and CD163-PE were stained for flow cytometry analysis. (**B**) Immunofluorescence staining of CD86 and CD163 was processed to evaluate the polarization of macrophages. Scale bar = 20 μm. (**C**) Quantitative real-time polymerase chain reaction (qRT-PCR) assays were processed to observe the influence of Akt2 inhibition on the expression of M1 phenotype-associated genes, including interleukin (IL)–1β, inducible nitric oxide synthase (iNOS), IL-6, and tumor necrosis factor–α (TNF-α). $P<0.05$ represents a significant difference between the indicated groups. (**D**) Western blot was processed to detect the expression of Arg-1. Cells were transfected with siNC and siAkt2, respectively, and exposed to IL-4 and IL-13 for 12 h. (**E**) Flow cytometry and (**F**) immunofluorescence staining were processed to analyze the polarization of macrophages. Scale bar = 20 μm. (**G**) qRT-PCR and (**H**) Western blot were processed to detect the expression of Arg-1. $P<0.05$ represents a significant difference.

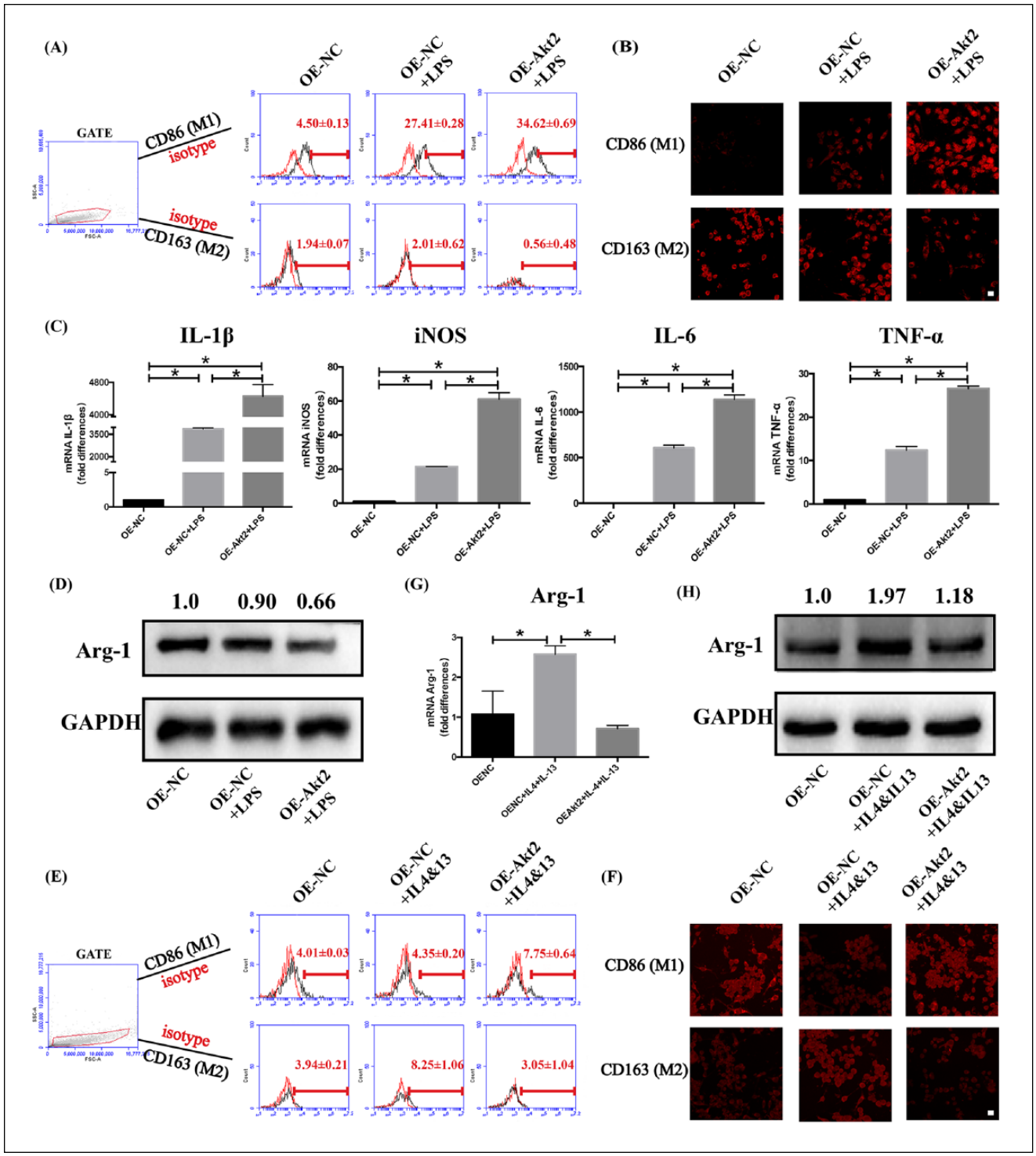

Figure 3. Overexpression of Akt2 promotes M1 polarization of macrophages. Akt2 was stably overexpressed in RAW 264.7 cells via lentivirus infection and exposed to lipopolysaccharide (LPS) for 12 h. (**A**) CD86-APC and CD163-PE were stained for flow cytometry analysis. (**B**) Immunofluorescent staining of CD86 and CD163 was processed to evaluate the polarization of macrophages. Scale bar = 20 μm. (**C**) Quantitative real-time polymerase chain reaction (qRT-PCR) assays were processed to observe the influences of Akt2 overexpression on the expression of M1 phenotype-associated genes, including interleukin (IL)–1β, inducible nitric oxide synthase (iNOS), IL-6, and tumor necrosis factor–α (TNF-α). $P<0.05$ represents a significant difference between the indicated groups. (**D**) Western blot was processed to detect the expression of Arg-1. Cells were exposed to IL-4 and IL-13 for 12 h. (**E**) Flow cytometry and (**F**) immunofluorescence staining were processed to analyze the polarization of macrophages. Scale bar = 20 μm. (**G**) qRT-PCR and (**H**) Western blot were processed to detect the expression of Arg-1. $P<0.05$ represents a significant difference.

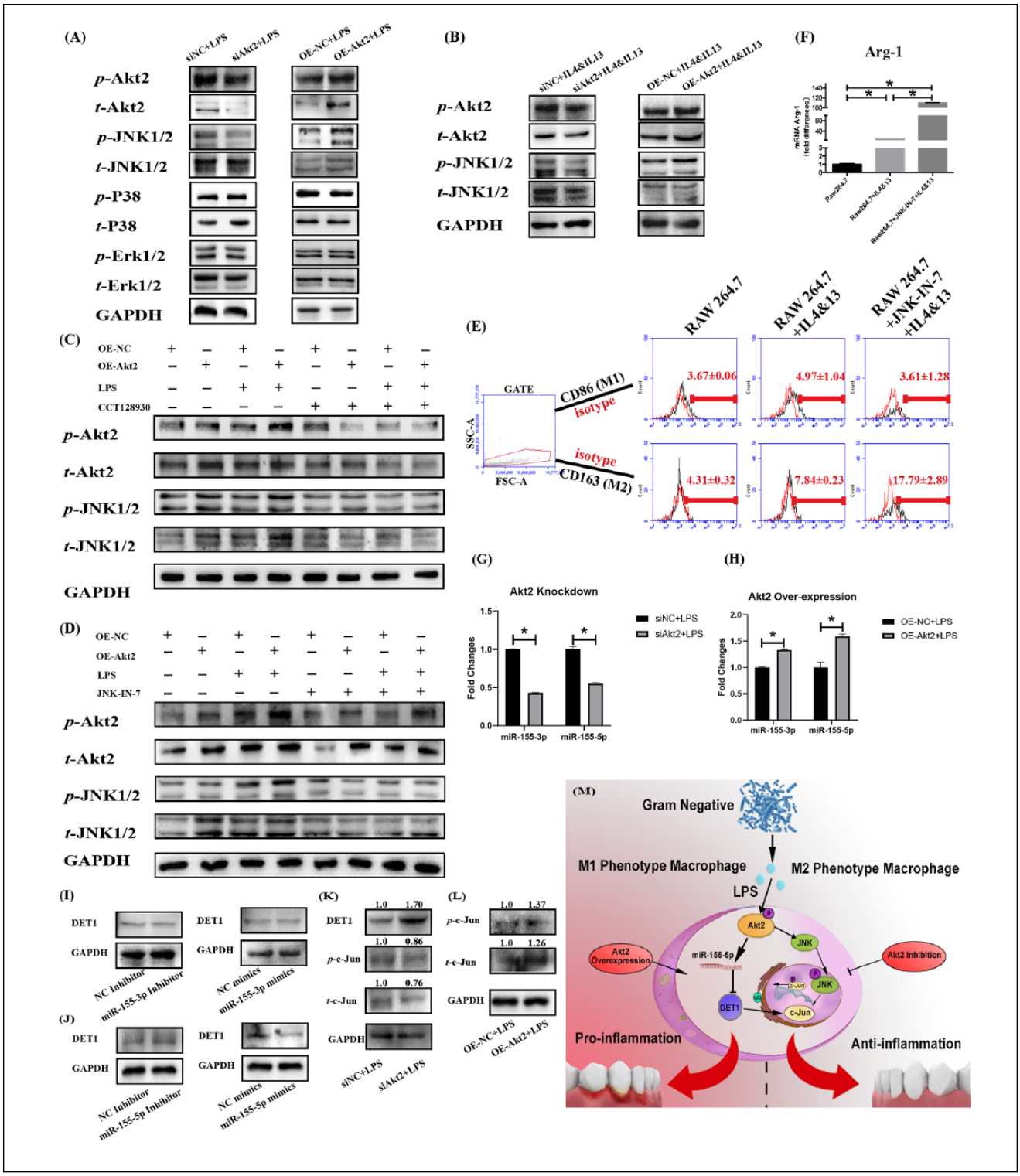

Figure 4. Akt2 regulates macrophage polarization through Akt2/JNK1/2/c-Jun and Akt2/miR-155/DET1/c-Jun signaling pathways. Western blot exhibited that both under the M1-promoting environment (**A**) and M2-promoting environment (**B**), overexpression of Akt2 resulted in higher expressions of *p*-JNK1/2 and *p*-Akt2, while inhibition of Akt2 could attenuate the phosphorylation levels of JNK1/2 and Akt2. Akt2 overexpression-induced signaling pathway alteration could be rescued by the Akt2 inhibitor (**C**) and JNK inhibitor (**D**). RAW 264.7 cells were pretreated with JNK-IN-7 and exposed to interleukin (IL)–4 and IL-13 for 12 h. (**E**) CD86-APC and CD163-PE were stained for flow cytometry analysis. (**F**) Quantitative real-time polymerase chain reaction (qRT-PCR) assay was processed to detect the expression of Arg-1. $P<0.05$ represents a significant difference. qRT-PCR assay revealed that Akt2 inhibition could attenuate the expression of both miR-155-3p and miR-155-5p (**G**), while Akt2 overexpression exhibited opposite effects (**H**). $P<0.05$ represents a significant difference. DET1 expression under the treatment of mimics and inhibitors of miR-155-3p (**I**) and miR-155-5p (**J**) was examined via Western blot. (**K**) Western blot revealed that Akt2 inhibition could increase the expression of DET1 and decrease the expression of both total and phosphorylated c-Jun. (**L**) Overexpression of Akt2 upregulated *p*-c-Jun. (**M**) Diagram illustrates that Akt2 could influence the polarization of macrophages and modulate inflammatory diseases such as periodontitis via the Akt2/JNK1/2/c-Jun and Akt2/miR-155/DET1/c-Jun signaling pathways.

addition, Akt2 overexpression increased the percentage of M1 phenotype macrophages (Fig. 3E, F). Compared with control, qRT-PCR and Western blot revealed higher expression of Arg-1 after M2 polarization, which was decreased after Akt2 overexpression ($P < 0.05$; Fig. 3G, H). The above data further demonstrated the effect of Akt2 on macrophage polarization.

Akt2 Regulates Inflammatory Environment via the JNK1/2 Signaling Pathway

To explore the underlying mechanism of Akt2 in macrophage polarization, Western blot was performed. The results showed that, under an M1 polarization-inducing environment, Akt2 inhibition could significantly decrease the expression of *p*-Akt2 and *p*-JNK1/2 (Fig. 4A, left row). In accordance with the results of Akt2 inhibition, Akt2 overexpression exhibited higher expression of *p*-Akt2 and *p*-JNK1/2, which indicated a more grievous inflammation status than others (Fig. 4A, right row). However, the influence of Akt2 on Erk1/2 and the P38 signaling pathway were not obvious (Fig. 4A). The expression of Akt2 and JNK1/2 signaling also has been investigated under the M2 polarization-inducing condition, and results were in accordance with LPS stimulation (Fig. 4B). These implied that Akt2 modulated the inflammatory environment via the JNK1/2 signaling pathway. CCT128930, an Akt2 inhibitor, could rescue the higher expression of *p*-Akt2 and *p*-JNK1/2 induced by Akt2 overexpression (Fig. 4C). The specificity of CCT128930 was investigated (Appendix Fig. 5). Moreover, the JNK inhibitor, JNK-IN-7, could only rescue the expression of *p*-JNK1/2 but had little influence on Akt2 expression and its phosphorylation (Fig. 4D). JNK inhibitor JNK-IN-7 was applied to validate whether JNK inhibition could facilitate macrophage M2 polarization. Flow cytometry and qRT-PCR results showed that IL-4 and IL-13 stimulation could increase Arg-1 expression as well as the percentage of M2 polarized macrophages, which was further increased via JNK-IN-7 treatment ($P < 0.05$; Fig. 4E, F). The results indicated that inhibition of JNK1/2 signaling could promote macrophage M2 polarization.

Akt2 Might Inhibit JNK Signaling via the miR-155-5p/DET1/c-Jun Signaling Axis

Under LPS stimulation, Akt2 inhibition resulted in a significant decrease in miR-155-3p and miR-155-5p expression ($P < 0.05$; Fig. 4G), while Akt2 overexpression exhibited a higher expression of miR-155-3p and miR-155-5p ($P < 0.05$; Fig. 4H). Western blot revealed that miR-155-3p mimics and miR-155-3p inhibitors failed to influence the expression of DET1 (Fig. 4I). However, miR-155-5p mimics clearly inhibited the expression of DET1, and miR-155-5p inhibitors could augment the expression of DET1 (Fig. 4J). Moreover, Akt2 inhibition significantly increased DET1 expression and decreased expression of total and phosphorylated (*p*) c-Jun, the target of DET1 (Fig. 4K). Overexpression of Akt2 upregulated *p*-c-Jun (Fig. 4L). Based on the above data, the regulatory mechanism was deduced in Figure 4M.

Akt2 Inhibition Facilitates M2 Phenotype Macrophage Infiltration and Prevents the Bone Loss of Ligature-Induced Periodontitis in Mice

Silk ligature-induced periodontitis of maxillary second molars has been constructed. Akt2 inhibitor CCT128930 was injected locally in the silk ligatures (ligature induced periodontitis [LIP]) + CCT128930 group every day. At 7 d postoperation, 3-dimensional reconstruction and X-ray revealed that the height of alveolar bone in the periodontitis group was significantly lower than in control and LIP + CCT128930 groups (Fig. 5A). The decrease of bone volume/total volume (BV/TV) in the LIP group was prevented via CCT128930 injection ($P < 0.05$; Fig. 5B). HE staining revealed significant alveolar bone loss in the LIP group, and the alveolar bone height and volume of the LIP + CCT128930 group were higher than those of the LIP group (Fig. 5C). Immunohistochemistry revealed that IL-1β expression was significantly higher in the LIP group, while CCT128930 could significantly decrease its expression. Although IL-10 expression in control and LIP groups showed little difference, CCT128930 injection could increase the expression of IL-10 (Fig. 5D). Macrophage infiltration could be observed in the periodontium of all groups. However, the numbers of macrophages in periodontitis were greater than in control and LIP + CCT128930 groups (Fig. 5E and Appendix Fig. 6). Immunofluorescence double-staining revealed significantly higher amounts of $CD86^+/CD68^+$ cells in the LIP group, followed by the LIP + CCT128930 group, and the control group exhibited the least $CD86^+/CD68^+$ cells (Fig. 5E). Compared with control and LIP groups, $CD163^+/CD68^+$ cells were significantly higher in the LIP + CCT128930 group (Fig. 5F). The results were further supported by immunostaining (Appendix Fig. 6). Moreover, immunofluorescence costaining of *p*-Akt2/CD68 and *p*-JNK/CD68 revealed that CCT128930 administration could attenuate the expression of *p*-Akt2 and *p*-JNK in macrophages (Fig. 5G, H and Appendix Fig. 7B and D). Immunohistochemistry also exhibited less expression of *p*-Akt2 and *p*-JNK in the LIP + CCT128930 group than in the LIP group (Appendix Fig. 7A, C). Collectively, the results imply that Akt2 inhibition could facilitate macrophage M2 polarization via JNK signaling and prevent the bone loss due to periodontitis.

Discussion

In this study, the novel Akt2/JNK1/2/c-Jun and Akt2/miR-155/DET1/c-Jun signaling pathways have been unraveled in the polarization of macrophages. Our initial research exhibited higher expression of *p*-Akt2 and *p*-JNK in inflammatory macrophages, which implied that these signaling pathways were involved in the M1 polarization of macrophages. Overexpression of Akt2 facilitates macrophage M1 polarization, while Akt2 inhibition could induce M2 polarization of macrophages. Rescue studies have been processed and indicated that Akt2 is upstream of JNK in macrophage polarization.

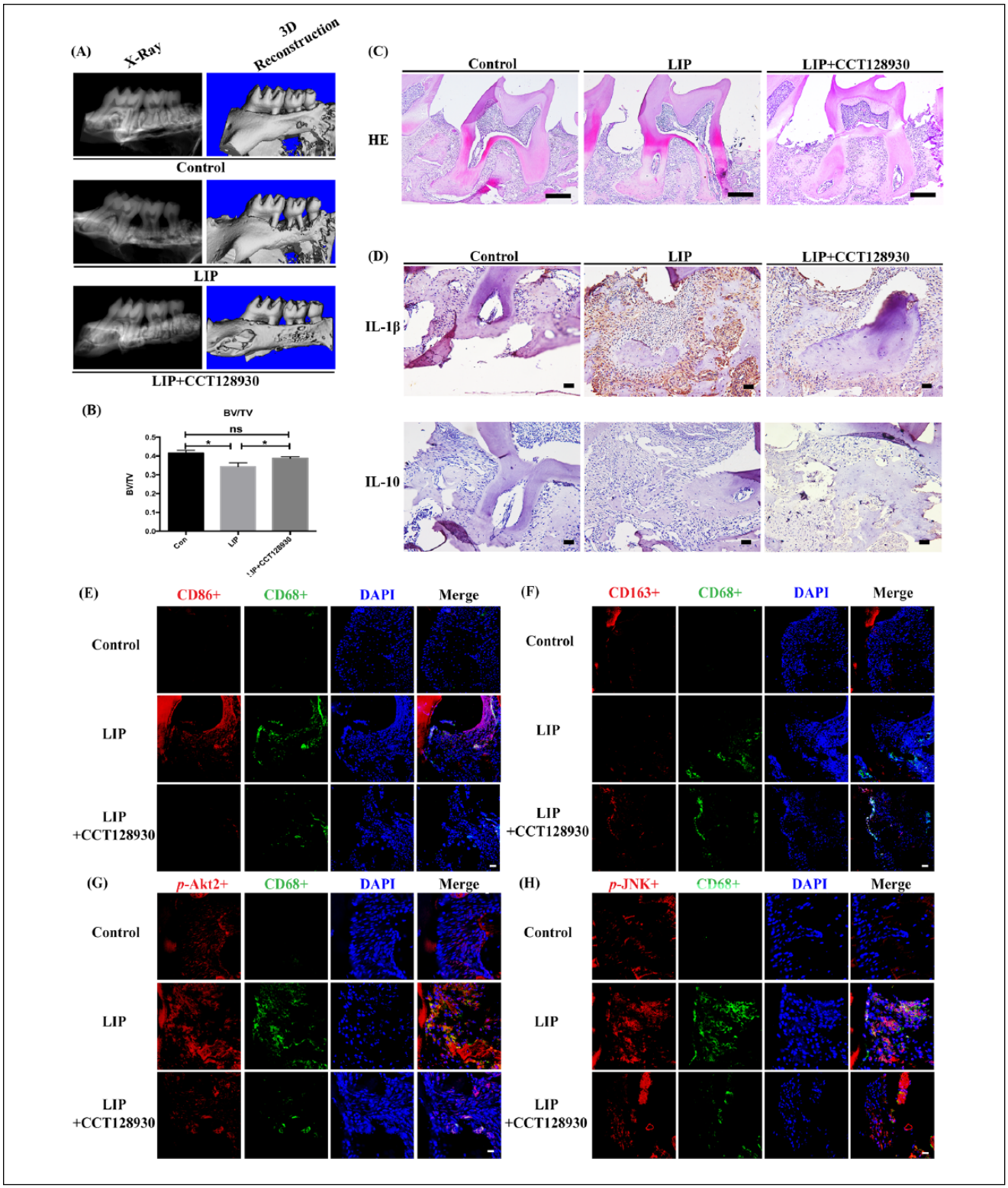

Figure 5. Akt2 inhibition facilitates M2 phenotype macrophage infiltration and rescues the bone loss of ligature-induced periodontitis in mice. (**A**) Representative X-ray and 3-dimensional reconstruction photos of control, ligature induced periodontitis (LIP), and LIP + CCT128930 groups. (**B**) Bone volume/total volume (BV/TV) of the above 3 groups is exhibited. (**C**) HE staining exhibited significant absorption of alveolar bones in the LIP group and less alveolar bone loss in the LIP + CCT128930 group. Scale bar = 500 μm. (**D**) Immunohistochemistry staining of interleukin (IL)–1β and IL-10 in control, LIP, and LIP + CCT128930 groups. Scale bar = 100 μm. Immunofluorescence colocalization staining of $CD86^+/CD68^+$ (**E**) and $CD163^+/CD68^+$ (**F**) cells in control, LIP, and LIP + CCT128930 groups. Scale bar = 50 μm. Immunofluorescence double staining of *p*-$Akt2^+/CD68^+$ (**G**) and *p*-$JNK^+/CD68^+$ (**H**) in control, LIP, and LIP + CCT128930 groups. Scale bar = 20 μm. Magnification: 200× (E, F) and 400× (G, H).

c-Jun, a key molecule of the JNK1/2 pathway, is downstream of JNK. Our new findings revealed that overexpression of Akt2 could activate *p*-JNK as well as *p*-c-Jun. Akt2 inhibition could attenuate the expression of *p*-JNK1/2 and *p*-c-Jun. In addition, inhibition of Akt2 could downregulate miR-155-5p expression, which in turn upregulates its target DET1 expression. DET1 is an E3 ubiquitin ligase, which can degrade c-Jun, finally leading to a decrease in phosphorylation of c-Jun and inactivation of JNK signaling.

Our results are in line with previous studies. In a colitis model, *Fusobacterium nucleatum* has been found to facilitate colitis via activating Akt2 signaling and regulating macrophage M1 polarization, which indicated that regulation of Akt2 signaling could be a potential strategy to prevent the progression of inflammation (Liu et al. 2019). Marsolier's research revealed that miR-155 could suppress DET1 expression and take part in the ubiquitination of c-Jun (Marsolier et al. 2013). The study by Arranz et al. (2012) demonstrated that Akt2 knockout could decrease miR-155 expression and attenuate its inhibition of C/EBPβ, which in turn activates STAT3 and induces macrophage M2 polarization in a dextran sulfate sodium–induced colitis model.

Periodontitis is one of the most prevalent diseases in dental clinics (Sun et al. 2017; Li et al. 2018; Renn et al. 2018). Macrophage polarization has been proposed to be involved in the progression of periodontitis. Controversies still exist about which kinds of macrophages are predominant in periodontitis. Through clinical samples investigation, our study revealed that the infiltration of both M1 and M2 macrophages in periodontitis was significantly higher than in the control group, while M1 phenotype macrophages were more dominant. Our data are in accordance with the most studies (Hussain et al. 2016; Yu et al. 2016; 2018). In addition, inducing macrophage M2 polarization proved to be effective in the treatment of periodontitis in this study. Collectively, the M1 macrophages should be the predominant phenotype in periodontitis. To our knowledge, the role of Akt2 in periodontitis has not been investigated before.

To confirm the effect of Akt2 on periodontitis, ligature-induced periodontitis was generated in mice, and Akt2 inhibitor CCT128930 was used to rescue the tissue absorption of periodontitis. Our results revealed that CCT128930 could significantly reduce the bone loss of periodontitis. Even though there was no significant difference in BV/TV between the control and LIP + CCT128930 groups, a small amount of bone loss could be observed. Considering the sterile silk was still visible during the treatment, continuous stimulation, including mechanical stimulation and bacterial stimulation, made it impossible to totally reverse the inflammatory environment and bone loss induced by periodontitis. However, the higher expression of IL-10 and $CD163^{+}$ macrophages made us realize the effects of Akt2 inhibition on the regulation of macrophage M2 polarization and its role on the treatment of periodontitis. Collectively, the study highlighted that Akt2 inhibition could induce M2 phenotype polarization of macrophages and Akt2 could be taken as a promising target in the treatment of periodontitis.

Conclusion

In this study, we deduced novel Akt2/JNK1/2/c-Jun and Akt2/miR-155-5p/DET1/c-Jun signaling pathways that can regulate macrophage polarization and modulate the periodontal proinflammatory environment to an anti-inflammatory status through inhibition of the Akt2 or JNK pathway. Specific inhibition of Akt2 could be a promising strategy for the treatment of periodontitis.

Author Contributions

X. Wu, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. Chen, contributed to data acquisition and analysis, drafted the manuscript; Y. Wang, Y. Gu, contributed to conception, design, and data analysis, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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References

Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, Margioris AN, Tsichlis PN, Tsatsanis C. 2009. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity. 31(2):220–231.

Arranz A, Doxaki C, Vergadi E, Martinez de la Torre Y, Vaporidi K, Lagoudaki ED, Ieronymaki E, Androulidaki A, Venihaki M, Margioris AN, et al. 2012. Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. Proc Natl Acad Sci USA. 109(24):9517–9522.

Cheng Y, Tian Y, Xia J, Wu X, Yang Y, Li X, Huang C, Meng X, Ma T, Li J. 2017. The role of PTEN in regulation of hepatic macrophages activation and function in progression and reversal of liver fibrosis. Toxicol Appl Pharmacol. 317:51–62.

Chukkapalli SS, Ambadapadi S, Varkoly K, Jiron J, Aguirre JI, Bhattacharyya I, Morel LM, Lucas AR, Kesavalu L. 2018. Impaired innate immune signaling due to combined Toll-like receptor 2 and 4 deficiency affects both periodontitis and atherosclerosis in response to polybacterial infection. Pathog Dis. 76(8):fty076.

Cole PA, Chu N, Salguero AL, Bae H. 2019. AKTivation mechanisms. Curr Opin Struct Biol. 59:47–53.

Czimmerer Z, Daniel B, Horvath A, Ruckerl D, Nagy G, Kiss M, Peloquin M, Budai MM, Cuaranta-Monroy I, Simandi Z, et al. 2018. The transcription factor STAT6 mediates direct repression of inflammatory enhancers and limits activation of alternatively polarized macrophages. Immunity. 48(1):75–90.e6.

Engelman JA, Luo J, Cantley LC. 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 7(8):606–619.

Gao W, Tang H, Wang D, Zhou X, Song Y, Wang Z. 2020. Effect of short-term vitamin D supplementation after nonsurgical periodontal treatment: a randomized, double-masked, placebo-controlled clinical trial. J Periodontal Res [epub ahead of print 20 Jan 2020]. doi:10.1111/jre.12719.

Garaicoa-Pazmino C, Fretwurst T, Squarize CH, Berglundh T, Giannobile WV, Larsson L, Castilho RM. 2019. Characterization of macrophage polarization in periodontal disease. J Clin Periodontol. 46(8):830–839.

Gheren LW, Cortelli JR, Rodrigues E, Holzhausen M, Saad WA. 2008. Periodontal therapy reduces arginase activity in saliva of patients with chronic periodontitis. Clin Oral Investig. 12(1):67–72.

Gordon S, Taylor PR. 2005. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 5(12):953–964.

Hussain QA, McKay IJ, Gonzales-Marin C, Allaker RP. 2016. Detection of adrenomedullin and nitric oxide in different forms of periodontal disease. J Periodontal Res. 51(1):16–25.

Kuijl C, Savage NDL, Marsman M, Tuin AW, Janssen L, Egan DA, Ketema M, van den Nieuwendijk R, van den Eeden SJF, Geluk A, et al. 2007. Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. Nature. 450(7170):725–730.

Li K, Lv G, Pan L. 2018. Sirt1 alleviates LPS induced inflammation of periodontal ligament fibroblasts via downregulation of TLR4. Int J Biol Macromol. 119:249–254.

Li N, Qin J, Lan L, Zhang H, Liu F, Wu Z, Ni H, Wang Y. 2015. PTEN inhibits macrophage polarization from M1 to M2 through CCL2 and VEGF-a reduction and NHERF-1 synergism. Cancer Biol Ther. 16(2):297–306.

Liu L, Liang L, Liang H, Wang M, Lu B, Xue M, Deng J, Chen Y. 2019. *Fusobacterium nucleatum* aggravates the progression of colitis by regulating M1 macrophage polarization via AKT2 pathway. Front Immunol. 10:1324.

Lu WL, Zhang L, Song DZ, Yi XW, Xu WZ, Ye L, Huang DM. 2019. NLRP6 suppresses the inflammatory response of human periodontal ligament cells by inhibiting NF-κB and ERK signal pathways. Int Endod J. 52(7):999–1009.

Mazza JE, Newman MG, Sims TN. 1981. Clinical and antimicrobial effect of stannous fluoride on periodontitis. J Clin Periodontol. 8(3):203–212.

Marsolier J, Pineau S, Medjkane S, Perichon M, Yin QY, Flemington E, Weitzman MD, Weitzman JB. 2013. Oncomir addiction is generated by a mir-155 feedback loop in theileria-transformed leukocytes. PLoS Pathog. 9(4):e1003222.

Michaud DS, Joshipura K, Giovannucci E, Fuchs CS. 2007. A prospective study of periodontal disease and pancreatic cancer in US male health professionals. J Natl Cancer Inst. 99(2):171–175.

Navarrete M, Garcia J, Dutzan N, Henriquez L, Puente J, Carvajal P, Hernandez M, Gamonal J. 2014. Interferon-γ, interleukins-6 and -4, and factor XIII-A as indirect markers of the classical and alternative macrophage activation pathways in chronic periodontitis. J Periodontol. 85(5):751–760.

Renn TY, Huang YK, Feng SW, Wang HW, Lee WF, Lin CT, Burnouf T, Chen LY, Kao PF, Chang HM. 2018. Prophylactic supplement with melatonin successfully suppresses the pathogenesis of periodontitis through normalizing RANKL/OPG ratio and depressing the TLR4/MyD88 signaling pathway. J Pineal Res. 64(3):e12464.

Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, Miyake T, Matsushita K, Okazaki T, Saitoh T, et al. 2010. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol. 11(10):936–944.

Sica A, Mantovani A. 2012. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest. 122(3):787–795.

Sun X, Mao Y, Dai P, Li X, Gu W, Wang H, Wu G, Ma J, Huang S. 2017. Mitochondrial dysfunction is involved in the aggravation of periodontitis by diabetes. J Clin Periodontol. 44(5):463–471.

Van Dyken SJ, Locksley RM. 2013. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. Annu Rev Immunol. 31:317–343.

Varin A, Gordon S. 2009. Alternative activation of macrophages: immune function and cellular biology. Immunobiology. 214(7):630–641.

Wang X, Li W, Song W, Xu L, Zhang L, Feng X, Lu R, Meng H. 2019. Association of CYP1A1 rs1048943 variant with aggressive periodontitis and its interaction with hyperlipidemia on the periodontal status. J Periodontal Res. 54(5):546–554.

Wu X, Zheng S, Ye Y, Wu Y, Lin K, Su J. 2018. Enhanced osteogenic differentiation and bone regeneration of poly(lactic-co-glycolic acid) by graphene via activation of PI3K/Akt/GSK-3β/β-catenin signal circuit. Biomater Sci. 6(5):1147–1158.

Yang J, Zhang L, Yu C, Yang XF, Wang H. 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark Res. 2(1):1.

Yang J, Zhu Y, Duan D, Wang P, Xin Y, Bai L, Liu Y, Xu Y. 2018. Enhanced activity of macrophage M1/M2 phenotypes in periodontitis. Arch Oral Biol. 96:234–242.

Yao Y, Xu XH, Jin L. 2019. Macrophage polarization in physiological and pathological pregnancy. Front Immunol. 10:792.

Yu S, Ding L, Liang D, Luo L. 2018. Porphyromonas gingivalis inhibits M2 activation of macrophages by suppressing α-ketoglutarate production in mice. Mol Oral Microbiol. 33(5):388–395.

Yu T, Zhao L, Huang X, Ma C, Wang Y, Zhang J, Xuan D. 2016. Enhanced activity of the macrophage M1/M2 phenotypes and phenotypic switch to M1 in periodontal infection. J Periodontol. 87(9):1092–1102.

Zhou LN, Bi CS, Gao LN, An Y, Chen F, Chen FM. 2019. Macrophage polarization in human gingival tissue in response to periodontal disease. Oral Dis. 25(1):265–273.

Zhuang Z, Yoshizawa-Smith S, Glowacki A, Maltos K, Pacheco C, Shehabeldin M, Mulkeen M, Myers N, Chong R, Verdelis K, et al. 2019. Induction of M2 macrophages prevents bone loss in murine periodontitis models. J Dent Res. 98(2):200–208.