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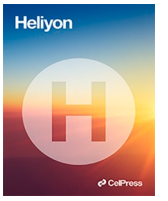

Research article

Porphyromonas gingivalis promote microglia M1 polarization through the NF-κB signaling pathway

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ABSTRACT

Background: Porphyromonas gingivalis (*P.gingivalis*) is associated with the onset of Alzheimer's disease (AD), but the underlying molecular mechanism is unclear. Neuroinflammation in the brain from the microglial immune response induces the pathological progression of AD. In this study, the roles and molecular mechanism of *P.gingivalis* in microglial inflammation *in vitro* were investigated.

Methods: In this study, a *P.gingivalis* oral administration mouse model was generated, and microglia were stimulated with *P.gingivalis in vitro*. The viability of the microglia after *P.gingivalis* treatment was evaluated through CCK-8 and live/dead cell staining. Inflammation in brain tissue after *P.gingivalis* treatment and the immune response of microglia *in vitro* were detected by RT–PCR, Western blotting and IF. Moreover, the RNA sequence was used, and the role of the NF-κB signalling pathway in microglial activation was analysed after *P.gingivalis* stimulation.

Results: The mRNA and protein levels of IL-6 and IL-17 were increased, and the expression of IL-10 was decreased in brain tissue after *P.gingivalis* oral administration. The viability of the HMC3 cells significantly decreased with 5% *P.gingivalis* after stimulation. The results of live/dead cell staining also showed the inhibitory effect of 5% *P.gingivalis* supplementation on cell viability. Moreover, 5% *P.gingivalis* supplementation increased the mRNA and protein levels of IL-6 and IL-17 and decreased IL-10 expression in HMC3 cells. *P.gingivalis* supplementation increased the mRNA and protein levels of iNOS and CD86 and decreased CD206 expression in HMC3 cells. RNA sequencing revealed that the NF-κB signalling pathway was involved in this process. Furthermore, p-P65 was upregulated and p-IKBα was downregulated in brain tissue and HMC3 cells after *P.gingivalis* stimulation, and an NF-κB signalling pathway inhibitor (QNZ) reversed the viability, M1 polarization and inflammatory factors of microglia in HMC3 cells *in vitro*.

Conclusions: In conclusion, *P.gingivalis* induced neuroinflammation in the brain, possibly through promotion of M1 polarization of microglia via activation of the NF-κB signalling pathway during the progression of AD.

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1. Introduction

Alzheimer's disease (AD) is the major cause of cognitive impairment, dementia and behavioural impairment and accounts for approximately 60–80 % of all dementia cases worldwide [1,2]. AD patients exhibit neuroinflammation consistent with infection, including microglial activation, inflammasome activation, complement activation, and related cytokine profiles. Infectious diseases caused by chronic periodontitis (CP) have been found to promote neuroinflammation in the brain and are postulated to be involved in AD [3,4]. Currently, many studies have shown that oral pathogenic microorganisms in the CP may play important roles in the pathological progression of AD.

CP is a complex infectious disease resulting from the inflammatory response of tooth support tissue caused by plaque-related bacteria. Periodontitis is the sixth most common disease in the world, affecting approximately 743 million people, with a high incidence rate of 11.2 % [5]. CP is also reported to cause several systemic diseases, such as nonalcoholic fatty liver disease, type 2 diabetes, coronary heart disease (CHD), and AD [6,7]. *Porphyromonas gingivalis* (*P.gingivalis*), the main pathogenic bacteria of the CP, impairs recognition in rats by activating microglia-mediated neuroinflammation [8], which is considered the hallmark of AD. Gingipains from *P.gingivalis* have been reported to be responsible for the migration and activation of microglia [9]. A prospective observational study reported that patients with active CP exhibited a notable decline in cognition over a 6-month period in AD patients [10]. Infection with *P.gingivalis* has been identified as a significant risk factor for the development of Aβ plaques, dementia, and AD [6,11]. *P.gingivalis* lipopolysaccharide (LPS) was detected in the brain tissue of AD patients and exhibited a strong microglial activation response in the brain [12,13]. Notably, *P.gingivalis* was detected in microglia [14]. Overactivation of microglia can lead to sustained neuroinflammation and contribute to the pathogenesis of neurodegenerative diseases [15]. All of these findings suggest that *P.gingivalis* maybe plays a role in the pathogenesis of AD through the microglial activation response.

In the central nervous system (CNS), microglia are the most common innate immune cells and play a central role in CNS tissue maintenance, injury response, and pathogen defence [4,16]. Microglia also participate in the development and shaping of neural circuits by engulfing and removing unnecessary neurons and synapses [16,17]. It is now well recognized that microglia exhibit functional plasticity and dual phenotypes, including the proinflammatory M1 phenotype and the anti-inflammatory M2 phenotype [9, 18]. The NF-κB signalling pathway plays an important role in the M1/M2 activation of microglia in neuroinflammation [19]. Inhibition of the NF-κB signalling pathway promotes microglial M2 polarization [20]. In the proinflammatory response, *P.gingivalis* upregulates the expression of genes involved downstream of the NF-κB signalling pathway in CP patients [21]. Additionally, *P.gingivalis* leads to oral epithelial cell death via the NF-κB signalling pathway [22]. *For example,* LPS activates the TLR2/4-mediated NF-κB signalling pathway, ultimately leading to an immune inflammatory response in microglia [23]. Therefore, we speculate that *P.gingivalis* infection promotes microglial M1 polarization by activating the NF-κB signalling pathway in neuroinflammation during the pathological progression of AD.

In this study, brain inflammation was detected after *P.gingivalis* oral administration *in vivo*. Moreover, cell viability, inflammatory factor levels, and microglial polarization after *P.gingivalis* stimulation *in vitro* were also analysed by IHC, IF, RT−PCR and Western blotting. The regulatory role of the NF-κB signalling pathway during microglial activation was also analysed.

2. Materials and methods

2.1. Animal model

Twelve (half female, half male) six-week-old C57BL/6 mice were purchased from Huachuang Sino Co., Ltd. (Jiangsu, Taizhou). All experimental equipment and procedures were examined and approved by the Institutional Animal Care and Use Committee of Shanghai Rat & Mouse Biotech Co., Ltd (No. SHDSYY-2022-3063-9). After adaptive feeding, the mice were randomly divided into two groups with equal numbers (half female, half male) (n = 6): the control group and the experimental group with *P.gingivalis* supplement. A total of 10^9 colony-forming units (CFU) of live *P.gingivalis* suspended in 200 μL of brain heart infusion (BHI, Shanghai Solarbio Science & Technology Co., Ltd.) broth was orally administered to each mouse once daily via a feeding needle while the control group was administered the same amount of BHI without *P.gingivalis*. During the experiment, the mice were allowed to eat and drink ad libitum and were weighed weekly. After 5 weeks, the mice were euthanized with CO2. Brain tissue was collected from 6 samples per group: three samples were fixed in 4 % paraformaldehyde, and others were stored at −80 °C for mRNA and protein extraction.

2.2. Pathological histology

Brain tissue was fixed in 4 % formalin (Shanghai Reith Biotechnology, Co., Ltd., China) and embedded in paraffin. Sections with a thickness of 4 μm were prepared before staining. Then, these sections were deparaffinized using xylene and rehydrated in a graded alcohol series (100 %, 90 %, 80 %, and 70 %), stained with hematoxylin-eosin staining (H&E, Shanghai Reith Biotechnology, Co., Ltd.) and periodic acid-Schiff (PAS, Shanghai Reith Biotechnology, Co., Ltd.) for histological examination, and then hydrated in a graded alcohol series (30 %–100 %). The results were quantified by bright-field light microscopy.

2.3. Brain tissue sample collection and treatment

Proteins were extracted from brain tissue with RIPA lysis buffer (200 μl, BioSharp, China), and the concentration of the harvested

lysate was determined with a BCA protein assay kit (Reith Biotechnology (Shanghai) Co., Ltd., China). Total RNA was isolated from the brain tissue by the TRIzol (Vazyme Biotech Co., Ltd., China) extraction method according to the manufacturer's protocol. Total RNA concentration was measured by a Nanodrop system (Thermo Fisher Scientific, United States). cDNA was reverse transcribed with PrimeScript RT Master Mix (Takara, Cat# RR036A) and subjected to qPCR using specific primers.

2.4. Bacterial cultures

The *P.gingivalis* strain (American Type Culture Collection, no. ATCC33227) was grown in Columbia Agar Base (Solarbio, Shanghai, China) under anaerobic conditions at 37 °C for 3 d. Subsequently, *P.gingivalis* was grown in BHI medium (Solarbio) supplemented with vitamin K (0.5 μg/mL), protohemin (5 μg/mL), yeast powder (1 mg/mL) and cysteine (0.05 %). The inoculum density, prepared in BHI broth, was adjusted to turbidity of 2 McFarland standard (6×10^8 colony-forming unit (CFU)/mL). The medium was then centrifuged at 3000 rpm for 5 min to collect the supernatant. The *P.gingivalis* supernatants were directly diluted with culture medium to the different concentration.

2.5. Cell culture and viability analysis

The HMC3 cell line (Cell Resource Center of the Shanghai Institutes for Biological Sciences (CRL-3304), China) is a normal human microglia cell line. The HMC3 cell line was cultured in α-MEM (Gibco, Grand Island, NY, USA) supplemented with 10 % FBS (Gibco) in an incubator at 37 °C with 5 % CO_2. Various assays including the CCK-8 and Live-Dead Cell Staining were utilized to assess cell viability. In the CCK-8 assay, HMC3 cells were seeded overnight in 96-well plates at a density of 5×10^4 cells/well. Subsequently, the culture medium was changed to medium with 10 % FBS containing different percentages of *P.gingivalis* (10^9/mL CFU) culture medium (from 1 % *P.gingivalis* supplement to 10 % *P.gingivalis* supplement) and incubated for 24 h. The total volume of this mixture was 100 μL, and the control group was supplemented with cell culture medium containing fresh BHI medium. 10 μL/well of CCK-8 solution (LAISI Biotechnology) was added and the HMC3 cells were incubated for 90 min at room temperature. Next, the 96-well plate was placed in a microplate spectrophotometer to measure the absorption value at 450 nm. For Live-Dead Cell Staining, cells were seeded at a density of 1×10^6 cells/well in 6-well plates and incubated overnight, following a 24-h incubation with a 5 % *P.gingivalis* culture medium. Cell viability was evaluated using the Live-Dead Cell Staining Kit (Vazyme Biotech, Shanghai, China), as per the manufacturer's guidelines. The resulting images were captured utilizing a Carl Zeiss LSM710 fluorescence microscope.

2.6. Immunohistochemical (IHC) and immunofluorescence (IF) staining

For IHC staining, the deparaffinized sections were subjected to heat-mediated antigen retrieval and blocked in H_2O_2 for 30 min. Then, the sections were blocked with 5 % BSA at room temperature and stained overnight with antibodies. Cells were fixed with 4 % paraformaldehyde (LaiSi) for 30 min, permeabilized with 0.1 % Triton-100 for 30 min, and subsequently rinsed twice with PBS. Following this, a blocking step was subjected with 5 % BSA at ambient temperature for 30 min. The cells were then incubated overnight at 4 °C with primary antibodies: anti-IL-6 (1:200; Abclonal, A1570, Shanghai, China), anti-IL-17 (1:200; Abclonal, A10587, Shanghai, China) and anti-IL-10 (1:200; Abclonal, A2171, Shanghai, China). A subsequent incubation was carried out with secondary antibodies (1:500; HRP, Rabbit Anti-Goat IgG, Proteintech, Wuhan, China) for 30 min and followed by triple rinsing with PBS at room temperature. Prior to coloration with a DAB kit (3,3′-diaminobiphenyl, LAISI Biotechnology), nuclei were stained using hematoxylin (LAISI Biotechnology). Imaging was facilitated using a Carl Zeiss LSM710 microscope.

For immunofluorescence (IF) staining, cells underwent fixation with 4 % paraformaldehyde (LAISI Biotechnology) for 10 min and permeabilization with 1 % Triton-100 for 30 min. Next, cells were incubated with Post-blocking, including cells anti–NF–κB (1:200; Abclonal, A2547, Shanghai, China), anti-iNOS (1:300; Abclonal, A3774, Shanghai, China), anti-CD86 (1:300; Abclonal, A16805, Shanghai, China), anti-CD206 (1:200; Cell Signal Technology, #24595, Danvers, MA, USA), anti-IL-6 (1:500; Abclonal, A1570, Shanghai, China), anti-IL-17 (1:200; Abclonal, A10587, Shanghai, China) and anti-IL-10 (1:200; Abclonal, A2171, Shanghai, China) at 4 °C overnight. Subsequently, samples were treated with Alexa Fluor 594-labeled (1:100; Proteintech) and Alexa Fluor 488-labeled secondary antibodies (1:100; Proteintech) for 2 h at ambient temperature. Nuclei visualization was achieved with DAPI staining, and imaging was conducted using a Carl Zeiss LSM710 fluorescence microscope.

2.7. Western blot

Protein samples underwent electrophoresis on polyacrylamide gels before being transferred to PVDF membranes (Merck Millipore, Temecula, CA, USA). The concentration of protein used for Westerning blotting is 200 μg. These membranes were then blocked using 5 % skim milk dissolved in PBS. Subsequently, they were incubated overnight at 4 °C with a series of primary antibodies including anti-IL-6 (1:1000; Abclonal, A1570, Shanghai, China), anti-IL-17 (1:1000; Abclonal, A10587, Shanghai, China), anti-IL-10 (1:1000; Abclonal, A2171, Shanghai, China), anti-CD86 (1:1000; Abclonal, A16805, Shanghai, China), anti-iNOS (1:1000; Abclonal, A3774, Shanghai, China), anti-CD206 (1:1000; Cell Signaling Technology, mAb#24595), anti–NF–κB (1:1000; Abclonal, A2547, Shanghai, China), anti-IKBα (1:1000; Abclonal, A1187, Shanghai, China), anti-p-IKBα (1:1000, Abclonal, AP0707, Shanghai, China) and anti-p–NF–κB (1:1000; Abclonal, AP0944, Shanghai, China) at 4 °C overnight. Following incubation, the membranes were rinsed with PBST (PBS with 0.1 % Tween-20) before being exposed to secondary antibodies at ambient temperature for 1 h. Followed by a triple wash with PBST, each lasting 5 min, the specific bands were then visualized using a Tanon-5200 Multi Chemiluminescent System (Tanon,

Shanghai, China) post-application of hypersensitive ECL luminescent liquid. Finally, the grayscale values of the bands were quantified utilizing ImageJ software.

2.7.1. Quantitative real-time PCR (RT–PCR)

After the extraction of total RNA, the resultant material was subjected to reverse transcription utilizing a PrimeScript RT Reagent Kit (TaKaRa, Dojindo, Kumamoto, Japan). The amount of RNA used for cDNA synthesis is 1 μg. This procedure was conducted

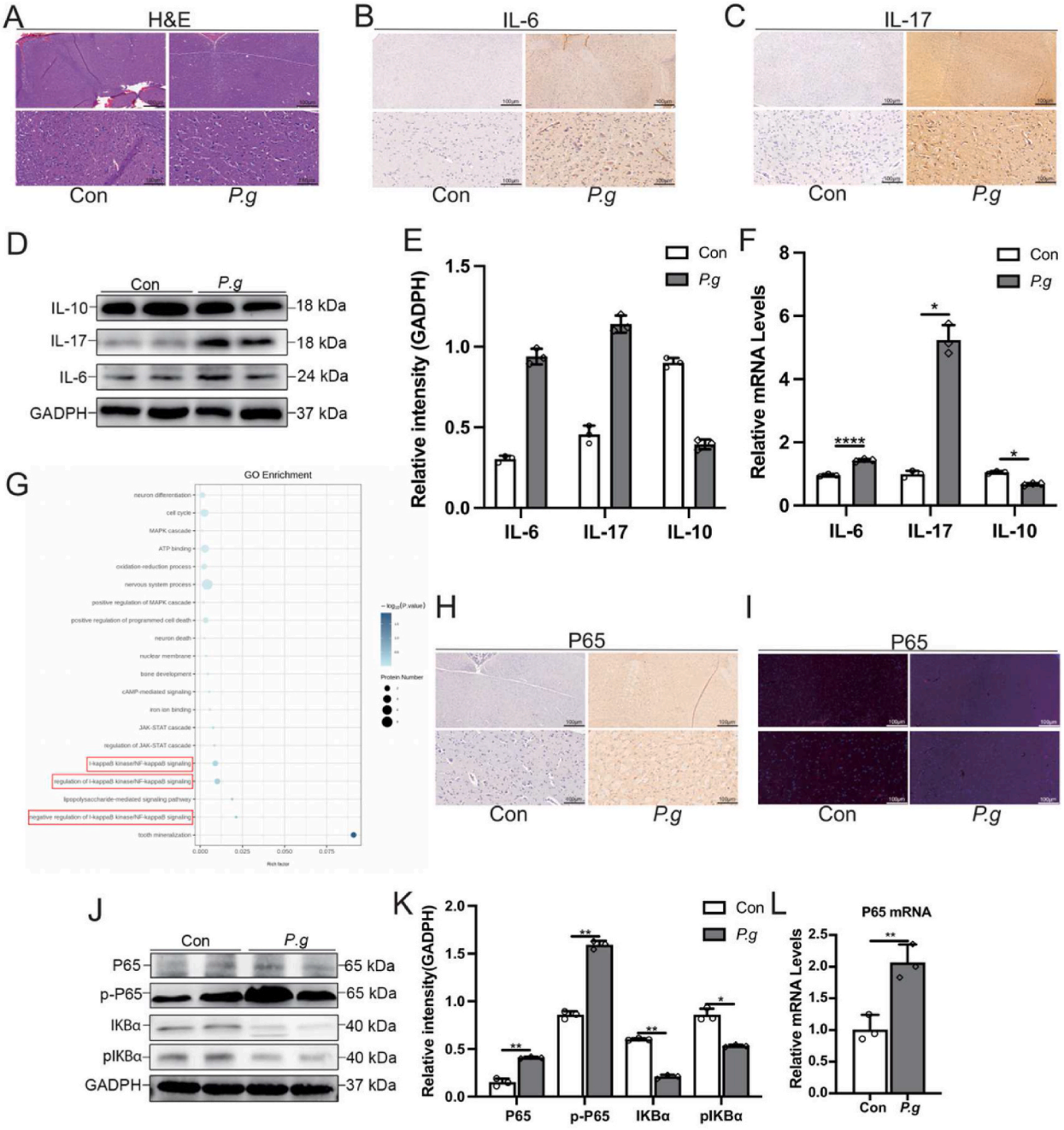

Fig. 1. Oral administration of *P.gingivalis* induces periodontitis and inflammation in brain. (A) H&E staining of inflammatory cell infiltration in brain sections. (B,C) IHC analysis of IL-6 and IL-17 expression in brain sections. (C) After *P.gingivalis* infection, body weight changes in the mice (D–F) *P.gingivalis* increased the protein and mRNA levels of IL-6, IL-17 and IL-10 *in vivo*. (G) Enrichment histogram of GO pathway in the *P.gingivalis* group compared with control group mice ($p < 0.05$). (H, I) IHC and IF results of P65 expression in brain sections. (J–L) *P.gingivalis* increased the protein and mRNA levels of P65 *in vivo*. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group).

sequentially at 37 °C for 15 min, 85 °C for 5 s, and maintained indefinitely at 12 °C. The qPCR was performed with HieffTM qPCR SYBR ® Green Master Mix (Yeasen Bio, Shanghai, China) in an ABI 7500 RT−qPCR System (Applied Biosystems, Foster City, CA, USA). The amplification and detection phases were carried out as follows: a 5-min hot start at 95 °C during the holding stage; 40 cycles of 10 s at 95 °C, 30 s at 60 °C during the cycling stage; and 15 s at 95 °C, 1 min at 60 °C, and 15 s at 60 °C during the melt curve stage. The relative gene expression levels were determined employing the $2^{-\Delta\Delta Ct}$ method.

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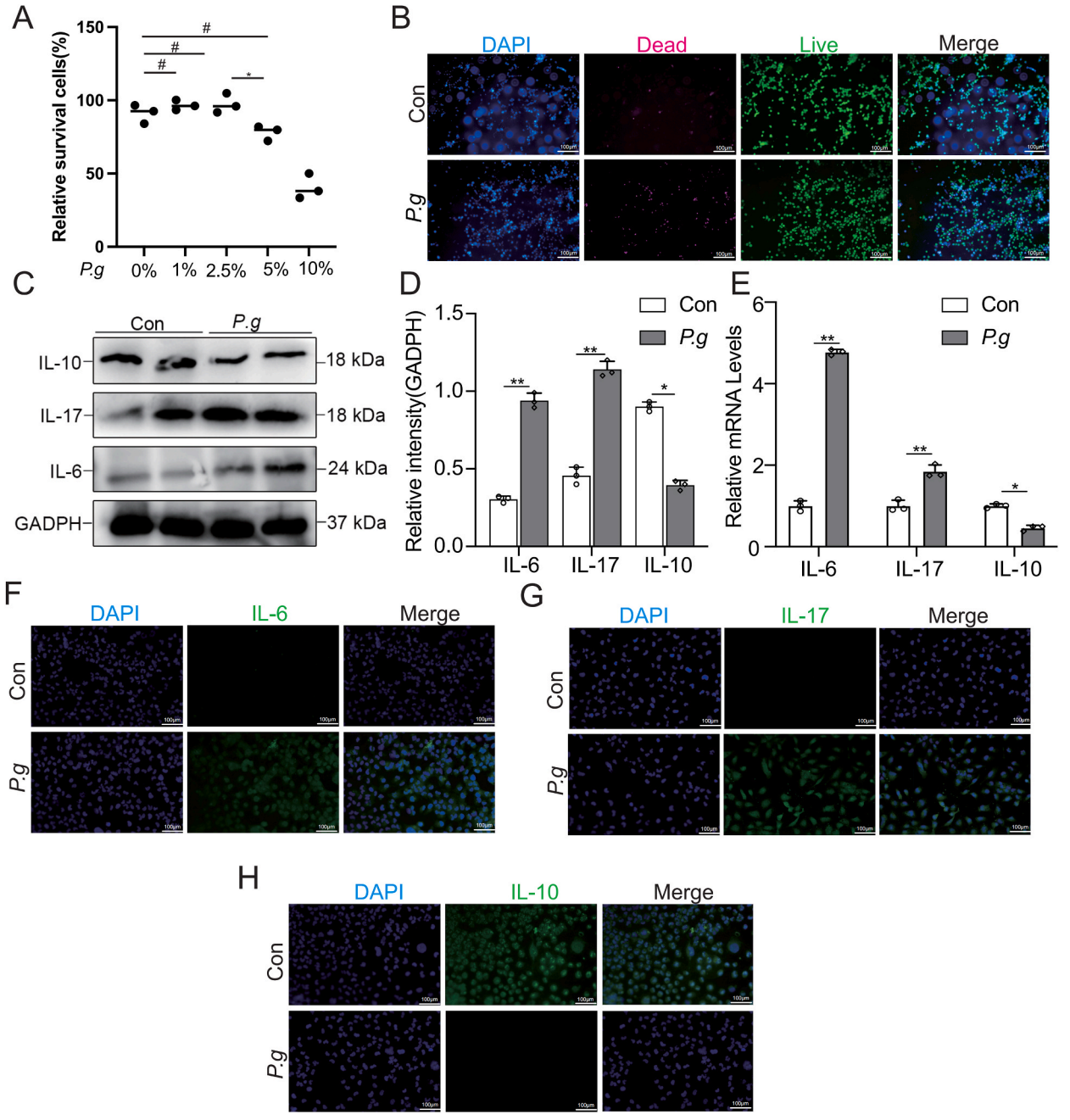

Fig. 2. ***P.gingiv***alis administration the production of pro-inflammatory cytokines. (A) CCK-8 analysis of MΦCM on HMC3 cells. (B) Staining of live/dead cells. (C–E) Protein and mRNA levels of IL-6, IL-17 and IL-10 on the microglia. (F–H) IF staining of the microglia cells for IL-6 (Alexa Fluor 488, green), IL-17 (Alexa Fluor 488, green), IL-10 (Alexa Fluor 488, green) and DAPI nuclei staining (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(*continued*)

Genes	Forward primer sequence (5′to 3′)	Reverse primer sequence (3′ to 5′)
Genes	Forward primer sequence (5′to 3′)	Reverse primer sequence (3′ to 5′)
IL-6	AACATGTGTGAAAGCAGCAAAGA	CTCTGGCTTGTTCCTCACTACTC
IL-17	CCATCTCATAGCAGGCACAAACT	GGATTTCGTGGGATTGTGATTCC
IL-10	AGCTCCAAGAGAAAGGCATCTAC	GTCTATAGAGTCGCCACCCTGAT
NF-κB	CTGGAACCACGCCTCTAGATATG	CAGCTGTTTCATGTCTCCTTGTG
CD86	CTGCTCATCTATACACGGTTACC	GGAAACGTCGTACAGTTCTGTG
iNOS	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
CD206	TCCGGGTGCTGTTCTCCTA	CCAGTCTGTTTTTGATGGCACT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

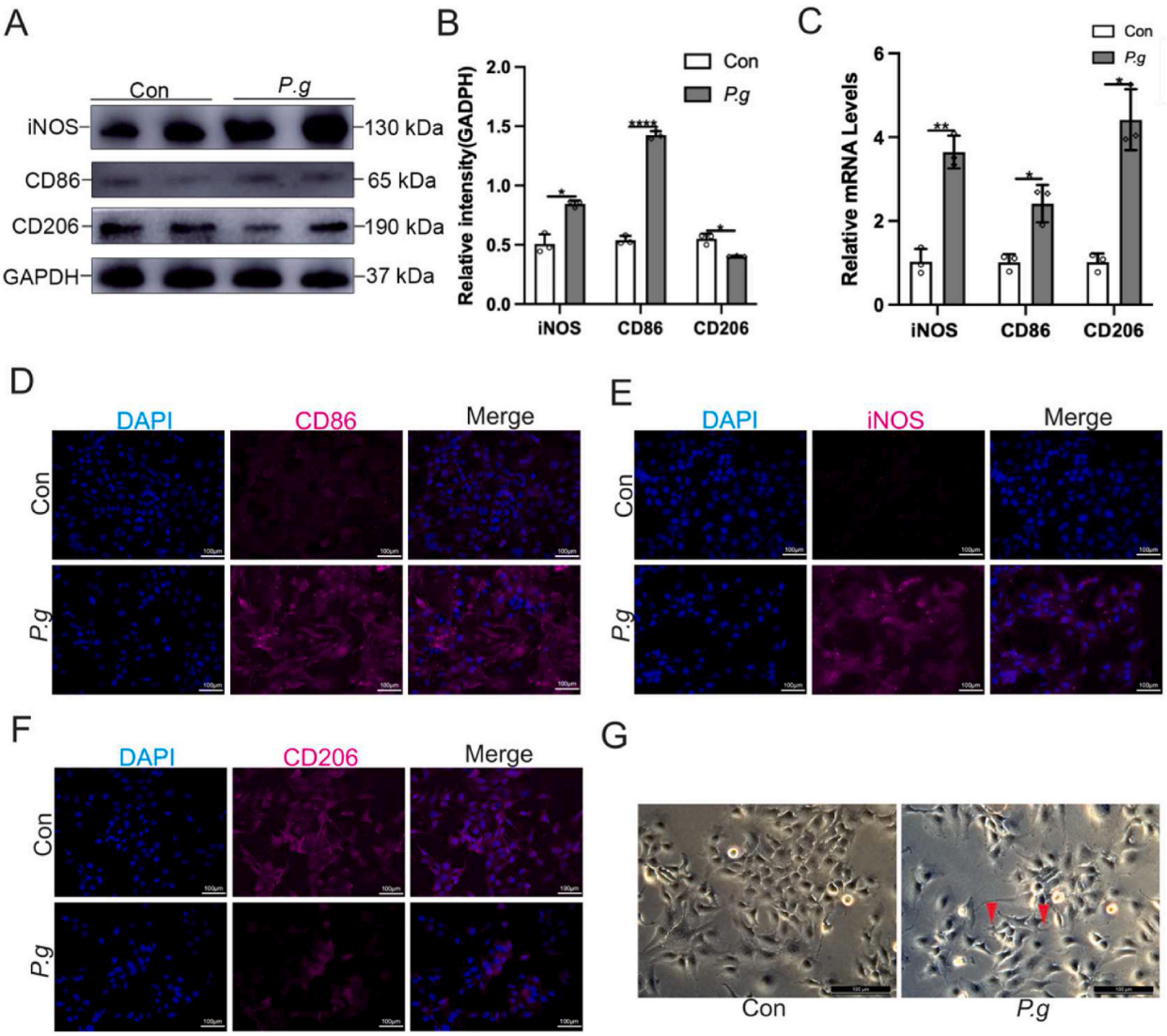

Fig. 3. P.gingivalis-administration microglia polarized to the M1 phenotype**.** (A–C) The expression of protein and mRNA showed that the expression level of CD86 and iNOS was high in the *P.gingivalis* group. (D–F) IF staining of HMC3 cells for CD86 (Alexa Fluor 594, red), iNOS (Alexa Fluor 594, red) and CD206 (Alexa Fluor 594, red) DAPI nuclei staining (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group). (G) Cellular morphology of HMC3 cell line in different group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

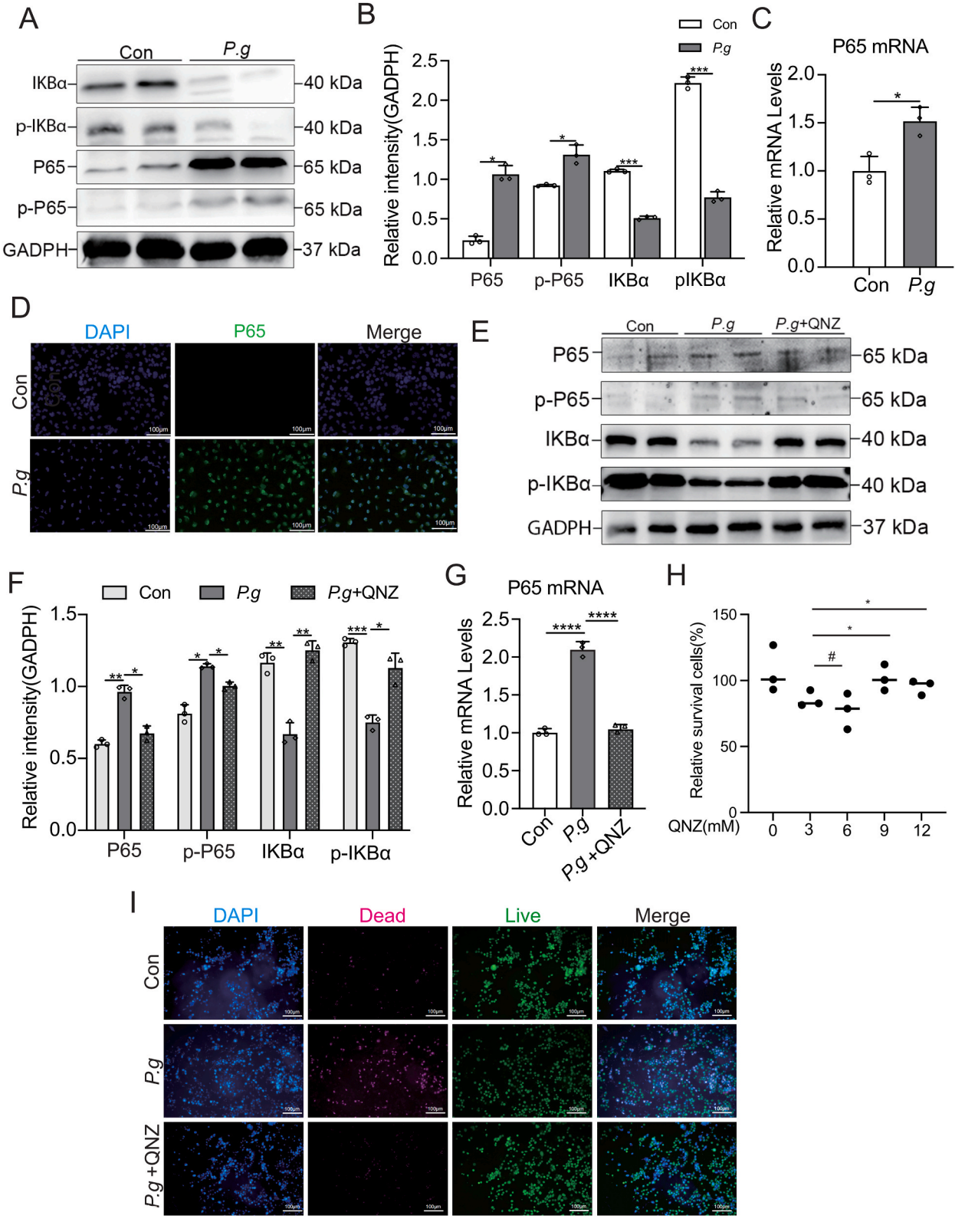

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Fig. 4. P.gingivalis administration activates the NF-κB pathway in HMC3 cells. (A–C) Protein and mRNA levels of NF-κB (P65) in HMC3 cells. (D) IF staining of SV40-MES-13 cells for NF-κB (P65) (Alexa Fluor 488, green) and DAPI nuclear staining (200 × magnification; scale bar 100 μm). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group). (E) CCK-8 analysis of HMC3 cells. (F) Staining of live/dead cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.8. The analysis of the NF-κB signaling pathway in microglia cell

HMC3 cells were seeded in 6-well plates at a density of 1×10^6 cells/well overnight. The cytotoxicity of QNZ (Sigma-Aldrich, Louis, MO, USA) was evaluated by CCK-8 assays. After 30 min of treatment with 9 μM QNZ, HMC3 cells were cultured with medium containing 10 % FBS and 5 % *P.gingivalis* supplement for 24 h. Then, HMC3 cell viability was detected as previously described. Moreover, the NF-κB signaling pathway, M1/M2 polarization and inflammatory factors (IL-6, IL-17 and IL-10) were detected by RT-qPCR and Western blotting.

2.9. Statistical analysis

All experiments were repeated at least three times, and the results were presented as the mean ± SD. Unpaired Student's t tests or ANOVA were performed using PRISM5 software for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

Oral *P.gingivalis* administration in mice induced the production of inflammatory cytokines and activation of the NF-κB signalling pathway in the brain.

H&E staining revealed inflammatory cell infiltration in the experimental group orally administered *P.gingivalis* compared with that in the control group, which indicated the occurrence of inflammation in the brain (Fig. 1A). IHC revealed a significant increase in the expression of IL-6 and IL-17 in the brain after *P.gingivalis* oral administration (Fig. 1B and C). Increased protein and mRNA levels of IL-6 and IL-17 were also observed in the brain tissue compared with those in the control group ($P < 0.01$). However, IL-10 was obviously downregulated in the brain in the *P. gingivalis* group, as shown by Western blot and RT−PCR ($P < 0.05$). All of these results showed that *P.gingivalis* activated the inflammatory response in the brain. To test the molecular mechanism by which *P.gingivalis* activates the inflammatory response in the brain, RNA sequencing was used to evaluate the NF-κB signalling pathway (Fig. 1G) ($P < 0.01$). Moreover, IF, IHC, RT−PCR and Western blotting revealed that the protein and mRNA levels of P65 were increased in brain tissue in the *P.gingivalis* group. Moreover, the level of p-P65 was increased, and the protein level of p-IKBα was decreased in the brain in the *P. gingivalis* group (Fig. 1H–L) ($P < 0.01$). Overall, *P.gingivalis* induced inflammation in the brain and activated the NF-κB signalling pathway.

3.1. P. gingivalis promoted proinflammatory cytokine production in HMC3 cells

To further identify the effect of *P.gingivalis* in the brain, we established a stimulation model by *P.gingivalis* supplements on HMC3 cells *in vitro*. After HMC3 cells were cocultured with different concentrations (from 1 % to 10 %) of *P.gingivalis*, cell viability significantly decreased compared with that in the control group ($P < 0.05$), especially in the 5 % and 10 % *P.gingivalis* supplementation groups ($P < 0.01$) (Fig. 2A). Therefore, a concentration of 5 % *P.gingivalis* supplements was used in the following study. The live-dead cell staining results showed that the number of dead cells (red) in the 5 % *P.gingivalis* supplementation group was much greater than that in the control group, while the number of live cells (green) showed the opposite trend (Fig. 2B). The mRNA and protein expression levels of IL-6 and IL-17 were increased, while the expression of IL-10 was significantly decreased in microglia after stimulation with *P. gingivalis* (Fig. 2C–H) ($P < 0.05$). These observations demonstrated that *P.gingivalis* induced proinflammatory responses in microglia *in vitro*.

3.2. P.gingivalis promoted microglial M1 polarization

To determine the role of *P.gingivalis* in the shifting of different microglial phenotypes, the expression of the CD86, iNOS and CD206 markers was detected. *P.gingivalis* increased the protein and mRNA levels of CD86 and iNOS in microglia but inhibited CD206 expression (Fig. 3A–C) ($P < 0.05$). The IF staining results showed the same trend as the mRNA and protein levels of CD86, iNOS and CD206 (Fig. 3D–F). Images of the HMC3 cells as well as of the different subtypes were taken to observe the morphological changes among the different groups. With tertiary and quaternary branching structures, the *P.gingivalis* group of HMC3 cells was highly branched (Fig. 3G). Taken together, these results suggested that *P.gingivalis* promoted M1 polarization in microglia *in vitro*.

3.3. P.gingivalis inhibited cell viability via activation of the NF-κB signalling pathway in HMC3 cells

The NF-κB signalling pathway in HMC3 cells was also detected after *P.gingivalis* treatment *in vitro*. Compared to those in the control group, increased protein and mRNA levels of P65 ($P < 0.05$), as well as p-P65, were clearly observed in microglia after P.gingivalis

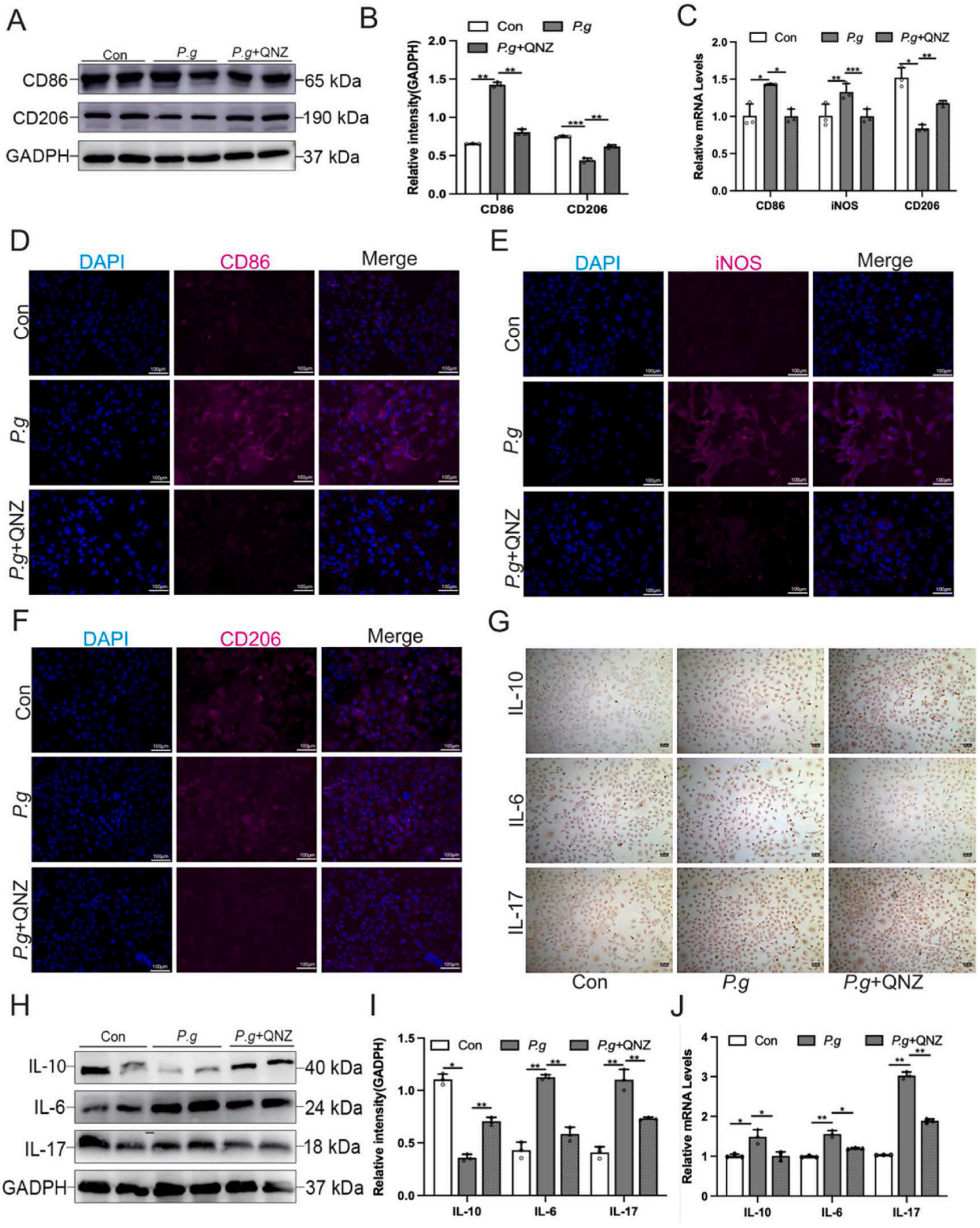

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Fig. 5. *P.gingivalis* administration caused M1 polarization via NF-κB pathway in HMC3 cells. (A–C) Protein and mRNA levels of CD86, iNOS and CD206 in HMC3 cells. (D) IF staining of CD86, iNOS and CD206 (Alexa Fluor 594, red) and DAPI nuclear staining (blue) in HMC3 cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group). (G) IHC analysis of IL-6, IL-17 and IL-10 expression in HMC3 cells. (H–J) Protein and mRNA levels of IL-6, IL-17 and IL-10 in HMC3 cells. Staining of live/dead cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, vs. the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

treatment, while the expression of p-IKBα was significantly decreased (Fig. 4A–C). Additionally, the IF staining results showed that P65 was transferred into the nucleus in HMC3 cells (Fig. 4D).

QNZ is an inhibitor of the NF-κB signalling pathway The changes in the expression of P65/p-P65 and IKBα/p-IKBα in microglia after *P.gingivalis* treatment *in vitro* were attenuated by QNZ (Fig. 4E–G) ($P < 0.001$), which showed that the NF-κB signalling pathway was inhibited. Furthermore, the decreased viability of microglia induced by *P.gingivalis* treatment was reversed by QNZ according to the CCK-8 and live/dead cell staining results (Fig. 4H and I) ($P < 0.01$). Therefore, these results suggested that *P.gingivalis* induced the death of HMC3 cells through the NF-κB signalling pathway.

3.3.1. P.gingivalis caused M1 polarization and inflammation via the NF-κB signalling pathway in HMC3 cells

Then, we explored the role of the NF-κB signalling pathway in M1 polarization and inflammation in HMC3 cells after *P.gingivalis* treatment. The increase in the protein and mRNA levels of CD86 and CD206 in HMC3 cells treated with *P.gingivalis* was reversed by QNZ (Fig. 5A–C) ($P < 0.01$). Furthermore, the changes in CD86, CD206 and iNOS expression in HMC3 cells after *P.gingivalis* treatment were reversed by QNZ (Fig. 5D–F), suggesting that *P.gingivalis* caused M1 polarization of HMC3 cells via the NF-κB signalling pathway. Moreover, the increases in the levels of the inflammatory factors IL-6 and IL-17 in HMC3 cells after *P.gingivalis* treatment were attenuated (Fig. 5G–J) ($P < 0.01$), indicating that *P.gingivalis* caused M1 polarization and inflammation via the NF-κB signalling pathway in HMC3 cells.

4. Discussions

Neuroinflammation of the CNS immune microenvironment, especially the activation of microglia, has been recognized as an important contributor to neurological dysfunction and cognitive impairment in AD. This study demonstrated that *P.gingivalis* induced an immune response to the microclimate and showed that *P.gingivalis* promoted the M1 polarization of microglia and proinflammatory factors by activating the NF-κB signalling pathway.

CP has been reported to be associated with the pathological progression of AD, where *P.gingivalis* may be the main factor. *P.gingivalis*, the most important pathogenic bacteria that causes periodontal disease, mediates inflammation and immune responses between the host through various cytopathic factors and causes lasting destruction of periodontal tissues. The concentration of *P. gingivalis* in saliva can reach 10^6/mL in patients with severe periodontitis [23]. Considering that the proportion of *P.gingivalis* in the oral flora is estimated to be 0.8 %, *P.gingivalis* is overrepresented in the subgingival plaques of periodontitis patients [24,25], and humans produce 1–1.5 L of saliva a day. Therefore, patients with severe periodontitis can swallow approximately 10^{13} of their *P.gingivalis* bacteria in one day. Considering the difference in body weight, we administered 10^9 *P.gingivalis* bacteria by oral administration of *P. gingivalis* in a mouse model to simulate patient conditions, and periodontitis induced by oral administration of *P.gingivalis* was also observed in our previous study [26]. *P.gingivalis* and its toxic protease gingipains, as well as *P.gingivalis* DNA and LPS, have been detected in the brain tissue of patients with AD [6,13]. Additionally, animal studies have indicated that *P.gingivalis* induces neuroinflammation in the brains of AD patients. Neuroinflammation, a central mechanism involved in neurodegeneration, as observed in AD, has been increasingly considered a hallmark of AD. *P.gingivalis.*, LPS-induced periodontitis successfully leads to neuroinflammation, which plays an important role in the cognitive impairment of C57BL/6 mice. *P.gingivalis*-induced periodontal infection was proposed to cause cognitive impairment by releasing proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-1β in the brain tissue of middle-aged mice [27]. Neuroinflammation, a common early pathological alteration in AD, is characterized by the development of a chronic inflammatory response in the brain. Recent studies have detected the presence of *P.gingivalis* and its virulence factors in AD-affected brains and have shown that *P.gingivalis*-lipopolysaccharide (LPS) contributes to neuroinflammation and neurodegeneration [1,2]. Previous studies revealed that *P.gingivalis* reduced the learning and memory abilities of wild-type rats, with reduced protein expression in neuronal nuclei in the hippocampus, which resulted in neuronal damage. For example, LPS activated microglia, degenerated neurons, and further increased tau hyperphosphorylation and the subsequent formation of neurofibrillary tangles, which can accelerate AD/ADRD pathogenesis [3]. The activated microglial brain contributes to inflammation in the brain, and activated microglia recognize non-pathogen-associated molecular patterns (PAMPs) on bacteria and their cellular debris [4,5]. The current view is that the inflammatory response in the AD brain is a downstream consequence of Abeita accumulation resulting in the activation of microglia, which initiates a proinflammatory cascade and leads to the local release of potentially neurotoxic substances such as cytokines, complement factors and reactive oxygen species. Interestingly, experimentally induced microbial infections and/or their virulence factors also appear to contribute to CNS inflammation and, in some cases, to Abeita deposition [6–8]. The immediate response to periodontal pathogens and their endotoxins is to activate local and systemic innate immune responses, leading to the recruitment of inflammatory cells (macrophages and B cells) that secrete cytokines [(interleukin (IL)-1, IL-6, tumour necrosis factor-alpha (TNF-a) and interferon-gamma (INF-γ)] [9–11]. The inability of the innate immune system to remove pathogens such as *P.gingivalis.* results in progressive local tissue destruction together with chronic systemic inflammation and a chronic systemic inflammatory response with the potential to damage distant organs such as the brain [12–15].

In the immune response in the central nervous system, microglia play a central role [16]. Microglia function in the elimination of waste products that accumulate in the brain. These cells also produce cytokines, such as IL-1, IL-6, tumour necrosis factor (TNF-), and reactive oxygen species, and they can induce neurodegeneration in AD, indicating that they may promote neurodegeneration. In this study, we focused mainly on the effect of *P.gingivalis* on inflammation in the brain. IL-6 and IL-17 were highly expressed, while IL-10 was inhibited in brain tissue after *P.gingivalis* oral administration *in vivo* (Fig. 1). Microglia are self-renewing immune cells of the CNS that arise from yolk sac foetal macrophages [28]. In response to stimuli, microglia undergo classical activation towards the M1 phenotype (which promotes inflammation) or neuroprotective activation and then develop towards the M2 phenotype (which is associated with anti-inflammatory functions). Additionally, proinflammatory cytokines are also released when inflammatory cells die, stimulating severe inflammation. Aβ and hyperphosphorylated tau are considered biomarkers of AD, both of which are linked to proinflammatory cytokines and *P.gingivalis*. In this study, microglial viability decreased with different concentrations of *P.gingivalis* supernatant, which suggested that *P.gingivalis* may promote inflammation by inducing microglial death. The expression of inflammatory factors such as IL-6, IL-10 and IL-17 in microglia after *P.gingivalis* treatment followed the same trend as that in brain tissue *in vivo* (Fig. 2), which showed that *P.gingivalis* may induce neuroinflammation in the brain by activating the microglial inflammatory response.

Neuroinflammation induced by bacterial or viral infection is considered a crucial part of the pathogenesis of AD, especially the activation of microglia. The activation of microglia involves two opposing phenotypes (M1 and M2): M1 macrophages release proinflammatory cytokines, and M2 microglia play neuroprotective roles [29]. Neuroinflammation attributable to microglial cell activity has been reported as one of the main pathophysiological events involved in the progression of various neurodegenerative disorders, such as AD, Parkinson's disease (PD), and multiple sclerosis. In this regard, recent findings have provided evidence that microglial polarization (shifting the M1 phenotype to the M2 phenotype) is a promising strategy for treating a range of neurodegenerative disorders. Moreover, it has been reported that the chronic nature of low-level infections, such as periodontitis and its products, could affect the defence of susceptible brains to the point at which microglia enter the M1 phase [9]. Moreover, healthy CNS maintenance and tissue repair rely on the balance of M1/M2 microglial activation, which plays a dual role in the pathogenesis of neuroinflammation: generating anti-Aβ antibodies and clearing amyloid plaques while contributing to Aβ production and accumulation. In our study, *P.gingivalis* upregulated the expression of M1 phenotype markers (CD86 and iNOS) and inhibited the expression of the M2 phenotype marker CD206 at the mRNA and protein levels *in vitro*, which suggested that *P.gingivalis* supernatant promoted neuroinflammation via microglial M1 polarization.

Further exploration of the molecular mechanisms involved revealed that the RNA sequence of brain tissue was strongly related to the NF-κB signalling pathway, a transcription factor that plays a crucial role in neuroinflammatory responses. The most frequently occurring inducible form of NF-κB consists of heterodimers of the P50/P65 subunits, which mainly function as transcriptional activators. Under normal conditions, P65 binds to IKBα (an endogenous inhibitor), which exists in an inactive form in the cytoplasm [30]. Once *P.gingivalis* LPS binds to the pattern recognition receptor TLR-2/4 on macrophages [31], phosphorylated IKBα (p-IKBα) is subsequently degraded, and P65 is subsequently phosphorylated and translocated from the cytoplasm to the nucleus, where it contributes to the expression of proinflammatory mediators such as IL-1β and IL-6. Interestingly, in our study, oral administration of *P.gingivalis* increased the expression of p-P65, while p-IKBα decreased this expression (Fig. 1). Moreover, *P.gingivalis* upregulated the expression of proinflammatory cytokines, as well as the phosphorylation of NF-κB proteins and nuclear translocation, in HMC3 cells *in vitro* (Fig. 4). Taken together, these findings suggested that *P.gingivalis* supplementation activated the NF-κB signalling pathway and M1 polarization of microglia. To explore the relationship between the NF-κB signalling pathway and M1 polarization of microglia, HMC3 cells were pretreated with QNZ (an inhibitor of the NF-κB signalling pathway) before *P.gingivalis* supplementation. The cell viability and M1 polarization marker results indicated that QNZ rescued the viability of microglia (Fig. 5) and inhibited M1 polarization. The changes in the levels of proinflammatory factors (IL-6 and IL-17) and an anti-inflammatory factor (IL-10) were also reversed upon exposure to QNZ. These results suggest that *P.gingivalis* induces an inflammatory response through M1 polarization in microglia via the NF-κB signalling pathway.

In conclusion, we demonstrated that *P.gingivalis* induced neuroinflammation in the brain after oral administration of *P.gingivalis* and promoted M1 polarization in microglia by activating the NF-κB signalling pathway. However, our research has several limitations that need to be considered. First, we lacked the detailed molecular mechanism of neuroinflammation *in vivo*. Second, our research focused primarily on investigating microglia-mediated neuroinflammation in AD, and the immune response of other immune cells to *P.gingivalis* in brain tissue needs to be further explored in the future.

CRediT authorship contribution statement

Xue Li: Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chao Yao:** Methodology. **Dongmei Lan:** Methodology. **Yurong Chen:** Methodology. **Yan Wang:** Conceptualization, Funding acquisition. **Shengcai Qi:** Conceptualization, Writing – review & editing, Formal analysis, Data curation, Funding acquisition.

Declaration of competing interest

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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