

# PPAR- $\alpha$ agonist pirinixic acid alleviates periodontitis-induced AD-like pathology via suppression of the IRF7-IFN- $\beta$ -IFITM3 axis in glial cells

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## Research Article

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# Abstract

**Background:** Periodontitis is increasingly recognized as a contributing risk factor for Alzheimer's disease (AD). Our previous research demonstrated that periodontitis activates brain glial cells and upregulates the innate immune protein interferon-induced transmembrane protein 3 (IFITM3), leading to  $\beta$ -amyloid ( $A\beta$ ) deposition. However, the underlying mechanisms remain unclear.

**Methods:** *In vitro*, primary mouse glial cells were treated as follows: Control, Porphyromonas gingivalis (Pg), Pg+siRNA-IRF7, and Pg+pirinixic acid group. Glial activation and interferon pathway gene expression were analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Immunofluorescence. Secreted IFN- $\beta$  was measured by ELISA. Neurons were then exposed to conditioned media from these glial cultures, and neuronal IFITM3 and  $A\beta$  levels were assessed via immunofluorescence. *In vivo*, a periodontitis model was established in C57BL/6J mice via silk ligation and Pg topical application, and the impact of periodontitis on intracranial neuroinflammation was assessed. Neuroinflammatory changes were compared with those of age-matched APP/PS1 mice. Hippocampal and cortical expression of IRF1-9, IFNs, IFITM3, and inflammatory genes was quantified by RT-qPCR. Finally, mouse periodontitis models were treated with PBS or pirinixic acid, and AD-like brain pathology was evaluated by RT-qPCR and immunohistochemistry.

**Results:** *In vitro*, Pg significantly upregulated IRF7, IFN- $\beta$ , and IFITM3 expression in glial cells, with markedly more pronounced effects observed in astrocytes than in microglia. Knockdown of *Irf7* or treatment with pirinixic acid effectively attenuated Pg-induced astrocyte activation, reduced IFN- $\beta$  levels in the culture supernatant, and subsequently suppressed neuronal IFITM3 upregulation and  $A\beta$  accumulation. Database analysis revealed that Toll-like receptor 4 (TLR4) is widely expressed in astrocytes, and Pg treatment significantly upregulated the gene levels of *Tlr4* and *Ticam1/Ticam2*. *In vivo*, periodontitis induced neuroinflammation and  $A\beta$  deposition in the brains of mice, with hippocampal expression patterns of PPAR- $\alpha$  and IRF7 closely resembling those observed in APP/PS1 transgenic mice. Furthermore, pirinixic acid treatment markedly ameliorated periodontitis-induced neuroinflammation.

**Conclusion:** These findings further substantiate the pathological link between periodontitis and AD, highlighting the importance of periodontitis prevention and treatment in AD management. Moreover, we identify the IRF7-IFN- $\beta$ -IFITM3- $A\beta$  axis as a novel molecular pathway and a potential therapeutic target for AD via the oral-brain axis.

## Background

Local chronic inflammation is a well-recognized risk factor for the onset and progression of distal organ complications. Periodontitis, a chronic inflammatory disease characterized by progressive destruction of the periodontal supporting tissues, results from complex dysbiotic interactions between subgingival microbiota and the host's innate and adaptive immune responses. This dysregulated immune response not only drives local inflammation and tissue destruction but also contributes to the pathogenesis of several systemic disorders [1].

Alzheimer's disease (AD), the most common neurodegenerative disorder in the elderly, involves inflammation, oxidative stress, and apoptosis, yet currently lacks effective therapeutic options [2]. Increasing evidence supports a strong pathological link between periodontitis and AD [3]. Epidemiological studies reveal that individuals with periodontitis, particularly severe cases, exhibit a significantly elevated risk of developing AD [4, 5]. Conversely, periodontal health in AD patients tends to deteriorate with disease progression, with higher prevalence and severity of inflammation correlating positively with cognitive decline [6, 7]. *Porphyromonas gingivalis* (*Pg*), a major periodontal pathogen, has been identified within brain tissues of AD patients [8]. Experimental studies demonstrate that intravenous administration of *Pg* in mice compromises blood-brain barrier (BBB) integrity via the Mfsd2a/Caveolin-1-mediated transcytosis pathway, thereby increasing permeability and facilitating the entry of bacteria and their virulence factors into the central nervous system [9]. Given these interconnections, the prevention and management of both periodontitis and AD represent urgent public health priorities in aging populations worldwide.

The innate immune response plays a crucial role in the onset and progression of AD [10, 11]. Interferon (IFN)-stimulated genes (ISGs) are consistently upregulated in the brains of AD patients and in multiple AD mouse models [12]. Among them, interferon-induced transmembrane protein 3 (IFITM3), an innate immune effector induced by type I IFN and well known for its antiviral activity, has recently emerged as a key contributor to AD pathology [13–15]. IFITM3 directly activates  $\gamma$ -secretase, promoting the aberrant cleavage of amyloid precursor protein (APP) into  $\beta$ -amyloid ( $A\beta$ ), thereby accelerating  $A\beta$  plaque deposition. Notably, genetic ablation of IFITM3 in AD mouse models substantially reduces cerebral  $A\beta$  accumulation and improves cognitive function. Furthermore, neuroinflammation further upregulates IFITM3 expression, thereby amplifying  $\gamma$ -secretase activity and enhancing the IFITM3- $A\beta$  axis, which may exacerbate AD progression [16].

Our previous work demonstrated that *Pg* triggers neuroinflammatory responses in the central nervous system, aberrantly activating the IFITM3- $A\beta$  axis and thereby inducing or aggravating AD-like pathological changes in the mouse brain [17]. However, the precise mechanisms by which periodontitis regulates the upstream IFN- $\beta$  signaling pathway remain poorly understood. Therefore, it is of crucial importance to identify therapeutic agents that inhibit IFITM3 expression, thereby disrupting the oral-brain axis-mediated neurodegeneration. High-throughput sequencing data suggest that pirinixic acid significantly downregulates IFITM3 expression [18], implying its new potential to alleviate AD pathology through modulation of this innate immune axis.

Pirinixic acid, a specific agonist of peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ), has demonstrated potent anti-inflammatory and tissue-protective properties across various disease models. In a rat periodontitis model, intraperitoneal administration of pirinixic acid effectively attenuated alveolar bone loss, suppressed adhesion molecule expression, and decreased neutrophil infiltration in periodontal tissues, collectively mitigating local inflammation [19]. In AD mouse models, pirinixic acid promoted astrocyte and microglial activation around  $A\beta$  plaques and enhanced their phagocytic capacity for  $A\beta$  clearance, ultimately improving  $A\beta$ -related pathology [20]. These findings suggest that PPAR- $\alpha$

agonists confer therapeutic benefits in both periodontitis and AD, thereby offering a plausible mechanistic connection between the two conditions. However, the precise pathway through which pirinixic acid inhibits periodontitis-induced IFITM3 activation has yet to be elucidated.

Toll-like receptors (TLRs) are evolutionarily highly conserved members of the pattern recognition receptor (PRR) family. They serve as core sensors of the innate immune system and act as the first line of defence in host protection. Studies have shown that TLR4 specifically recognizes lipopolysaccharide (LPS) from Gram-negative bacteria [21]. Intraperitoneal injection of *Pg*-LPS significantly upregulates TLR4 expression in the mouse cerebral cortex, while exerting no significant effect on TLR2 or TLR3 [22]. Specific inhibition of TLR4 effectively blocks *Pg*-LPS-induced cognitive decline. However, the above evidence is derived primarily from acute LPS stimulation models. Whether *Pg* under chronic periodontitis conditions activates central neuroinflammation through TLR4-mediated recognition and activation remains unclear.

Members of the IFN regulatory factor (IRF) family, which serve as key transcriptional regulators of type I IFN and ISGs expression, have been implicated in neurodegenerative processes, notably IRF3, IRF4, and IRF7 [23–26]. However, whether periodontitis influences IRF activity remains unexplored. Given the established role of type I IFN signaling in regulating IFITM3 expression, we sought to determine whether pirinixic acid alleviates AD-like pathology through an IRF-dependent pathways.

This study demonstrates that periodontitis activates IFITM3 through IRF7-mediated IFN- $\beta$  signaling pathway. The PPAR- $\alpha$  agonist pirinixic acid alleviates periodontitis-induced AD-like pathology by suppressing the *Pg*-activated IRF7-IFN- $\beta$ -IFITM3-A $\beta$  signaling axis. Together, these findings uncover a novel mechanistic link between periodontitis and AD and propose a promising therapeutic strategy aimed at preventing and treating periodontitis-associated AD-like neurodegeneration.

## Methods

### Animals

Sixteen-week-old C57BL/6J and 28-week-old APP/PS1 mice were obtained from the SPF Laboratory Animal Center of Dalian Medical University. All experimental procedures were conducted in accordance with ARRIVE guidelines and were approved by the Laboratory Animal and Ethics Committee of Dalian Medical University (AEE23140). The mice were housed under standard conditions with ad libitum access to food and water, maintained at constant temperature and humidity, and subjected to a 12-hour light/dark cycle.

### *Porphyromonas gingivalis*

*Porphyromonas gingivalis* W83 (*Pg* W83, ATCC, USA) was cultivated anaerobically using a previously described method [17]. *Pg* W83 were grown on agar medium (Hope Bio-Technology, China)

supplemented with 36g/L agar powder (Solarbio, China), 500 mg/L yeast extract (Solarbio), and 5% defibrinated goat blood (Solarbio). Incubation was carried out at 37°C in an atmosphere consisting of 80% N<sub>2</sub>, 10% CO<sub>2</sub>, and 10% H<sub>2</sub>.

## Periodontitis model

Sixteen-week-old C57BL/6J mice were randomly allocated into two groups: a control group (C57) and a periodontitis group (C57-P). Periodontitis was induced by a combination of silk ligation and repeated topical application of *Pg* every 2 days, whereas the control group received topical applications of PBS only. The model was established over a 12-week experimental period. Following successful model establishment, periodontitis mice were randomly divided into two groups. The experimental group received intraperitoneal injection of pirinidic acid (PD + Pir group, 1 mg/kg) once every two days for one month, while the periodontitis control group received intraperitoneal injection of PBS (PD group).

## Cell culture

Primary mouse glial cells and neurons were isolated and cultured following an established protocol [27]. Briefly, hippocampal tissues were aseptically dissected from postnatal day 4 (P4) mice under a stereomicroscope. The collected tissues were then digested with 0.025% trypsin (Solarbio) at 37°C for 30 minutes. After digestion, the resulting cell suspension was seeded at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> and maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

Glial cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Solarbio). Neuronal cells were maintained in Neurobasal medium (Gibco) containing 2 mmol/L GlutaMAX (Gibco), 1% penicillin-streptomycin (Solarbio), and 1X B-27 supplement (Gibco). After cell attachment, glial cultures were assigned to three experimental groups: i) control group, ii) *Pg*-treated group, and iii) *Pg* plus pirinixic acid co-treated group. Treatments were applied by direct addition to culture medium, using *Pg* (MOI = 1:50) and/or pirinixic acid (10 μM, MCE, USA) as indicated.

BV2 immortalized mouse microglial cells and C6 immortalized rat astrocytes were obtained from the China Center for Type Culture Collection (CCTCC, China). Both cell lines were maintained in DMEM medium containing 10% FBS, 1 mM sodium pyruvate (Solarbio), 1% penicillin-streptomycin, and 1% MEM non-essential amino acids (Gibco). All cultures were maintained at 37°C under a humidified 5% CO<sub>2</sub> atmosphere.

## siRNA transfection

When cell reached 70% confluence, transfection was performed using mouse *Irf7* siRNA (Cyagen Biosciences, China) and the Lipo3000 reagent (Invitrogen, China) in accordance with the manufacturer's

protocol. The transfection efficiency was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The siRNA sequences used were listed in Table S1.

## Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from mouse hippocampus and cortical tissues as well as cultured cells using Trizol reagent (Sparkjade, China). According to the manufacturer's protocol, RNA was reverse-transcribed into cDNA with a commercial kit (Genestar, China). Quantitative PCR was subsequently performed using 2×RealStar Fast SYBR qPCR Mix (Genestar) (Primer sequences were listed in Table S2). *Gapdh* was used as the internal control, and relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method.

## Immunohistochemical (IHC) staining

Mouse mandibular tissues were fixed in 4% paraformaldehyde (PFA) and subsequently decalcified in 10% EDTA for two weeks. Following decalcification, the tissues were paraffin-embedded and sectioned into 5 µm-thick slices. Mouse brain tissues were fixed in 4% PFA for 24 hours, then similarly processed for paraffin embedding and sectioning at a thickness of 7 µm.

For histological evaluation, tissue sections were stained with Hematoxylin and Eosin (H&E). Immunofluorescence staining was performed on rehydrated sections. After blocking with normal serum for 1 h at room temperature, sections were incubated overnight at 4°C with the following primary antibodies: anti-GFAP antibody (ab68428, abcam, Britain), anti-IFITM3 antibody (ab15592, abcam), anti-IBA1 antibody (ab283346, abcam), and anti-Aβ antibody (Proteintech, 25524-1-AP), anti-IRF7 antibody (ET1610-89, HUABIO, China), anti-Neun (abcam, ab104224). The following day, the corresponding secondary antibodies were incubated at room temperature, including Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077, Abcam), Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113, Abcam), Goat Anti-Rabbit IgG H&L (Cy3 ®) preadsorbed (ab97075, Abcam), and Goat Anti-Rat (A23340, Abbkine, China). Nuclei were counterstained with DAPI and then photographed using a Zeiss microscope (Germany).

For immunohistochemical analysis of Aβ, sections were rehydrated, treated with hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity, and then incubated with anti-Aβ antibody at 4°C. Detection was performed the following day using a secondary antibody kit (PV-9001, ZSGB-BIO, China) followed by DAB (ZSGB-BIO) development. Nuclei were lightly counterstained with hematoxylin (Solarbio). Stained sections were visualized and imaged under a Zeiss microscope.

## Novel object recognition test (NORT)

The NORT was conducted over three consecutive days using a standard protocol. On day 1, mice were acclimatized to the empty testing arena for 10 minutes. The following day (day 2), two identical cube-shaped objects (A + A) were placed in the arena, and mice were allowed to explore them for 10 minutes. On day 3, one cube was replaced with a novel cylindrical object (A + B), and exploration was recorded for 5 minutes. All sessions were video-monitored. Object exploration time was automatically quantified using a video tracking system. The recognition index was calculated accordingly.

## Sequencing databases analysis

In this study, the periodontitis microarray dataset GSE16134, the hippocampal RNA-seq dataset GSE168137 from AD mice, and the brain Single-nucleus RNA sequencing (SnRNA-seq) dataset GSE260461 from patients with AD and healthy controls were retrieved from the Gene Expression Omnibus (GEO) database. The brain SnRNA-seq dataset SCP1375 from normal and AD mouse models were obtained from the Single-Cell Portal (SCP) database. The parahippocampal gyrus (PHG) proteomics dataset syn20801188, derived from patients with AD and healthy controls, was obtained from Synapse (Syn) database. Differential expression analysis was performed using the limma package in R (version 4.3.1), including normalization, standardization of gene expression values, and statistical testing. Genes with a Benjamini–Hochberg-adjusted false discovery rate (FDR) < 0.05 and an absolute  $\log_2$  fold change ( $|\log_2\text{FC}| \geq 0.5$ ) were considered significantly differentially expressed. Volcano plots were generated based on normalized expression values to visualize the distribution of differentially expressed genes (DEGs). The snRNA-seq data analysis in this study was performed following the methods and using the publicly available code described in reference [28]. The analytical pipeline included data quality control, normalization, gene filtering, dimensionality reduction, clustering, cell type annotation, and differential gene expression analysis. All analyses were conducted using the R programming environment.

## Statistical analysis

All statistical analyses were performed with using SPSS (26.0), and graphs were generated using GraphPad Prism 8.0 software. Data in this study conformed to a normal distribution. Differences between the two groups were assessed using independent samples t-tests, while comparisons across multiple groups were conducted by one-way ANOVA. A  $P < 0.05$  was considered statistically significant.

## Results

### **Pg activates glial cells to secrete IFN- $\beta$ and upregulate the neuronal IFITM3-A $\beta$ pathway**

Our previous findings demonstrated that *Pg* triggers neuroinflammation in glial cells and upregulates IFITM3, thereby promoting A $\beta$  processing—a response associated with the IFN family. To investigate this further, we treated mouse primary glial cells with *Pg* and observed changes in IFN expression. The upregulation of *Ifitm3* was positively correlated with increased expression of type I interferons, *Ifn-a* and *Ifn- $\beta$* , whereas *Ifn- $\gamma$*  expression was downregulated. Notably, *Ifn- $\beta$*  gene expression showed the most

pronounced induction (Fig. 1A). Consistent with this, the protein level of IFN- $\beta$  was also elevated in the supernatant of *Pg*-treated glial cells (Fig. 1B).

Immunofluorescence staining confirmed that *Pg* treatment activated both astrocytes and microglia (Fig. 1C, Supplementary Fig. 1A). Furthermore, the proportion of cells co-expressing IFITM3 and GFAP was significantly increased, and elevated APP/A $\beta$  protein levels in GFAP-positive astrocytes (Supplementary Fig. 1B).

Interestingly, direct treatment of neuronal cells with *Pg* did not alter IFITM3 or APP/A $\beta$  protein levels (Fig. 1D-F). However, when neurons were exposed to conditioned medium from *Pg*-treated glial cells, both IFITM3 and APP/A $\beta$  protein levels were markedly increased (Fig. 1G-I). Collectively, these results suggest that *Pg* upregulates the neuronal IFITM3-A $\beta$  pathway primarily by activating glial cells and inducing the secretion of IFN- $\beta$ .

### ***Pg* -upregulated IRF7 mediates IFN- $\beta$ production in glial cells**

Given that IRFs are key transcription factors regulating type I interferon expression, we examined the effect of *Pg* on *Irf1-Irf9* in glial cells. *Pg* treatment upregulated the mRNA levels of *Irf1*, *Irf6*, *Irf7*, and *Irf9*, with *Irf7* showing the most pronounced increase (Fig. 2A). Immunofluorescence staining further confirmed that *Pg* elevated IRF7 protein expression in both astrocytes and microglia, with a more prominent effect observed in astrocytes (Fig. 2B, C). Consistent results were obtained in astrocyte and microglial cell lines, where *Pg*-induced increases in *Ifitm3* and *Ifn- $\beta$*  positively correlated with elevated mRNA levels of *Irf7* (Supplementary Fig. 2A, B). Notably, the changes in these genes were less pronounced in microglial cells compared to astrocytes, suggesting that *Pg* may predominantly exert its effects through astrocytes (Supplementary Fig. 2A, B). These results indicate that *Pg* likely activates the IRF7-IFN- $\beta$  pathway in glial cells, particularly in astrocytes.

Knockdown of *Irf7* using siRNA (siIRF7) significantly attenuated the *Pg*-induced upregulation of both *Ifitm3* and *Ifn- $\beta$*  mRNA (Fig. 2D, E) and reduced the secretion of IFN- $\beta$  protein (Fig. 2F). Immunofluorescence analysis further demonstrated that siIRF7 alleviated *Pg*-induced astrocyte activation and decreased IFITM3 protein levels (Fig. 2G, H). Additionally, siRNA-mediated knockdown of IRF7 significantly reduced the number of IBA1-positive microglia activated by *Pg* treatment (Supplementary Fig. 2C, D). Moreover, it suppressed the *Pg*-induced upregulation of APP/A $\beta$  protein levels in GFAP-positive astrocytes (Supplementary Fig. 2E, F). siIRF7 effectively lowered IRF7 protein expression specifically in astrocytes and microglial cells (Fig. 2I, J, Supplementary Fig. 2G, H). Moreover, conditioned medium from glial cells treated with *Pg* in the presence of siIRF7 failed to elevate IFITM3 and APP/A $\beta$  protein levels in neurons (Fig. 2K-N).

Analysis of sequencing data from healthy controls and patients with AD revealed that astrocytes and microglia are the primary cell populations expressing TLR4. Moreover, TLR4 was the only Toll-like receptor widely expressed in astrocytes (Fig. 3A-C). Protein-protein interaction analysis using the STRING database identified interactions between TLR4 and IRF7, primarily mediated by TICAM1 and

TICAM2, which are key components of the MyD88-independent pathway (Fig. 3D). Further analysis of the sequencing data showed that *IRF7*, *IFITM3*, *TICAM1*, and *TICAM2* expression levels were elevated in the brains of patients with AD compared with healthy controls (Fig. 3E). Consistent with the above findings, *Pg* treatment significantly increased the mRNA expression of *Tlr4*, *Ticam1*, and *Ticam2* in primary glial cells (Fig. 3F). Similar changes were observed in astrocyte cell lines, whereas no significant alterations were detected in microglial cell lines (Supplementary Fig. 2A, B). These findings indicate that *Pg* activates glial cells via TLR4, resulting in the upregulation of IRF7 within those cells, which subsequently promotes IFN- $\beta$  release and enhances the neuronal IFITM3-A $\beta$  axis.

## Periodontitis upregulates the cerebral IRF7-IFN- $\beta$ -IFITM3 signaling axis

We next established a periodontitis model in C57BL/6J mice by ligating the first maxillary molar with silk sutures followed by local application of *Pg* (Supplementary Fig. 3A). After 12 weeks, mice with periodontitis exhibited alveolar bone loss and cognitive decline (Supplementary Fig. 3B-D), accompanied by neuroinflammation in the hippocampal and cortical regions, as evidenced by elevated expression of *Il-17*, *Il-1 $\beta$* , *Il-6*, and *Tnf- $\alpha$*  (Supplementary Fig. 3E). Increased levels of A $\beta$  precursor (APP) and A $\beta$  deposition were also observed in the hippocampus (Supplementary Fig. 3F-H).

In the periodontitis group, hippocampal protein levels of IFITM3 and APP/A $\beta$  were elevated, with higher expression specifically localized to activated GFAP-positive astrocytes (Fig. 4A, B). IRF7 protein levels in the hippocampus were also increased (Fig. 4C). The periodontitis-induced upregulation of hippocampal *Ifitm3*, *Ifn- $\alpha$* , and *Ifn- $\beta$*  mirrored the pattern seen in APP/PS1 mice. In contrast, *Ifn- $\gamma$*  expression was decreased in the periodontitis group but significantly elevated in APP/PS1 mice (Fig. 4D). Furthermore, consistent upregulation of IFN regulatory genes, including *Irf1*, *Irf2*, *Irf3*, *Irf7*, and *Irf9*, was detected in the hippocampi of both periodontitis and AD model mice (Fig. 4E).

Analysis of hippocampal tissues from normal and AD model mice using the GEO database GSE168137 revealed significantly increased mRNA expression of *Tlr4*, *Ifitm3*, *Irf1*, *Irf5*, *Irf7*, *Irf8*, and *Irf9*, *Ticam1*, *Ticam2* in AD mice compared with controls (Fig. 4F). Consistent with these findings, analysis of three additional datasets demonstrated elevated expression of *IRF7* and *IFITM3* in astrocyte from both human and mouse AD brains relative to controls. Furthermore, bulk RNA-seq data from the human PHG revealed similar alterations, accompanied by changes in the IFN-related pathway genes (Table S3).

Corroborating these central nervous system findings, analysis of sequencing data from periodontal tissues of healthy individuals and patients with periodontitis demonstrated significantly elevated expression of *TLR4*, *IFITM3*, *IRF1*, *IRF2*, *IRF7*, *IRF8*, and *TICAM2* in periodontitis samples compared with controls, whereas no significant changes were observed in the expression of *TICAM1* and *IFN- $\beta$*  (Fig. 4G). The consistency of these gene expression patterns across distinct pathological conditions in the oral cavity and brain further supports the existence and significance of the oral-brain axis.

Taken together, these findings support the involvement of interferon signaling in periodontitis-induced AD-like cerebral pathology and underscore the critical role of the *Pg*-induced IRF7-IFN- $\beta$ -IFITM3 signaling axis in this process.

### **PPAR- $\alpha$ agonist pirinixic acid attenuates *Pg*-induced upregulation of the IFITM3-A $\beta$ pathway**

Given prior reports that the PPAR- $\alpha$  agonist pirinixic acid (Pir) alleviates both periodontitis and AD pathology, we investigated its potential role in our model. Querying the Comparative Toxicogenomics Database (CTD) predicted that PPAR- $\alpha$  activation downregulates IFITM3. We thus hypothesized that Pir suppresses IFITM3 by inhibiting the IRF7-IFN pathway.

Consistent with this, *Ppar- $\alpha$*  mRNA levels were downregulated in the hippocampal and cortical tissues of both periodontitis and AD model mice (Supplementary Fig. 4A). In primary mouse glial cells challenged with *Pg*, co-treatment with Pir significantly attenuated the *Pg*-induced upregulation of *Ifitm3*, *Ifn- $\beta$* , *Irf7*, and *Tlr4* mRNA (Fig. 5A). Immunofluorescence analysis confirmed that Pir inhibited *Pg*-induced glial activation and markedly reduced IFITM3 and APP/A $\beta$  protein levels, with the effect being most prominent in astrocytes (Fig. 5B, C; Supplementary Fig. 4B, C). Furthermore, Pir suppressed the *Pg*-upregulated protein level of IRF7, leading to a significant decrease in the proportion of GFAP<sup>+</sup>IRF7<sup>+</sup> cells and IBA1<sup>+</sup>IRF7<sup>+</sup> cells (Fig. 5D, E; Supplementary Fig. 4D). Pir also reduced the level of IFN- $\beta$  protein in the conditioned medium from *Pg*-challenged glial cultures (Fig. 5F).

Mirroring the results from IRF7 knockdown, the conditioned medium from *Pg*-challenged glial cells, which normally upregulates IFITM3 and APP/A $\beta$  in neurons, failed to do so when glial cells were co-treated with Pir (Fig. 5G, H). These data collectively indicate that the PPAR- $\alpha$  agonist pirinixic acid inhibits the *Pg*-induced activation of the neuronal IFITM3-A $\beta$  pathway by modulating the IRF7-IFN- $\beta$  axis in glial cells.

## **Pirinixic acid blocks the IRF7-IFN- $\beta$ -IFITM3 axis and alleviates periodontitis-induced AD-like pathology and cognitive decline**

We next assessed the therapeutic effect of pirinixic acid *in vivo* using our mouse periodontitis model (Fig. 6A). H&E staining showed that Pir treatment partially restored periodontal bone structure (Fig. 6B). In the NORT, pir-treated mice exhibited a significantly higher number of contacts and longer exploration time with the novel object compared to the periodontitis control group (Fig. 6C, D). RT-qPCR analysis demonstrated that Pir administration reduced the periodontitis-induced upregulation of hippocampal *Ifitm3*, *Irf7*, and *Ifn- $\beta$*  mRNA levels (Fig. 6E).

Immunofluorescence staining revealed that Pir treatment decreased the protein levels of GFAP and IFITM3 in the hippocampal and cortical regions, accompanied by a reduction in the number of GFAP<sup>+</sup>IFITM3<sup>+</sup> co-expressing cells (Fig. 6F, I). APP protein levels in GFAP<sup>+</sup> cells were also lower in the Pir-treated group than in the periodontitis group (Fig. 6G, J). Of course, we also observed that Pir

suppressed the expression of APP/A $\beta$  protein, which is highly expressed in mouse brain neurons induced by periodontitis (Fig. 6H, K). Furthermore, fluorescent staining confirmed that Pir suppressed the elevated IRF7 protein levels in the brain tissues of periodontitis mice (Fig. 6L, M).

Collectively, these *in vivo* findings demonstrate that pirinixic acid inhibits the IRF7-IFN- $\beta$  axis, thereby attenuating the cerebral IFITM3-A $\beta$  pathway, neuroinflammation, and cognitive deficits induced by periodontitis.

## Discussion

Therapeutic development for AD remains challenging due to the advanced stage at which most patients are diagnosed, underscoring the need for early intervention strategies. Periodontitis, a chronic inflammatory condition that shares epidemiological and pathological features with AD, has emerged as a plausible modifiable risk factor. Here, we demonstrate that experimental periodontitis induces a neuroinflammatory profile in mice that closely mirrors that of AD models, reinforcing a direct pathological link between the two diseases.

Our previous work demonstrated that *Pg* induces neuroinflammation and upregulates IFITM3 in the brain, thereby promoting neuronal A $\beta$  deposition. However, the underlying molecular mechanisms remain incompletely understood. The present study identifies the IRF7-IFN- $\beta$  signaling axis as a critical upstream regulator of IFITM3, suggesting that IRF7 may function as a key transcription factor linking peripheral inflammation and central neuroinflammation. Consistent with this notion, previous studies have shown that IRF7 expression is significantly upregulated during the transition of microglia from an anti-inflammatory M2-like phenotype to a pro-inflammatory M1-like phenotype. Moreover, knockdown of IRF7 suppresses M1 marker expression and STAT1 phosphorylation, underscoring its central role in promoting pro-inflammatory polarization [29]. Additionally, LPS stimulation induces co-upregulation of IRF7 and IFITM3 in specific astrocyte subsets [24], further supporting the broad involvement of IRF7 in glial activation.

Screening of the CTD identified pirinixic acid, a PPAR- $\alpha$  agonist, as a compound capable of reducing IFITM3 expression [18]. In the present study, we observed a significant downregulation of *Ppar- $\alpha$*  gene expression in the brains of both periodontitis model mice and AD model mice. This shared molecular alteration suggests that PPAR- $\alpha$  may function as a key regulatory node linking periodontitis to AD pathology.

Previous studies have shown that PPAR- $\alpha$  is highly expressed during embryonic development and remains moderately expressed in cognitive-related brain regions, such as the hippocampus and medial prefrontal cortex, in adulthood [30]. In contrast, *Ppar- $\alpha$*  knockout mice exhibit impaired spontaneous alternation behavior [31], indicating its essential role in maintaining cognitive function. Notably, PPAR- $\alpha$  agonists, including pirinixic acid and gemfibrozil, have been reported to ameliorate AD-related pathology through multiple mechanisms, including modulation of APP expression, promotion of ADAM10-mediated non-amyloidogenic APP processing, activation of autophagy-lysosomal pathways to enhance A $\beta$

clearance, and suppression of NF- $\kappa$ B-mediated neuroinflammatory responses [32–36]. Our findings further reveal that downregulation of PPAR- $\alpha$  may relieve its inhibitory effect on the IRF7-IFN- $\beta$ -IFITM3 signaling axis, thereby establishing a modifiable molecular link between neuroinflammation and A $\beta$  deposition. This mechanistic insight expands current understanding of the role of PPAR- $\alpha$  in AD and identifies a potential therapeutic target for populations at risk of periodontitis-associated AD.

Astrocytes play multifaceted and complex roles in the onset and progression of AD, encompassing both their physiological functions in maintaining central nervous system homeostasis and their context-dependent contributions under pathological conditions [37, 38]. Previous studies have demonstrated that astrocytes are capable of generating A $\beta$  within the brain [39–41]. Consistent with these observations, our results show that *Pg* stimulation and periodontitis upregulate IFITM3 and promote A $\beta$  production in astrocytes. These findings suggest that astrocytes may influence neuronal function through intrinsic A $\beta$  production, thereby contributing to the initiation and progression of AD pathology. Furthermore, we observed differential responses among glial cell types following periodontal pathogen stimulation. *Pg* predominantly induced upregulation of IRF7 and IFN- $\beta$  in astrocytes, suggesting that astrocytes may play a more prominent role in signal amplification and propagation in periodontitis-induced neuroinflammation. This finding provides new insight into the functional specialization and coordination of glial cells in AD pathology.

TLR4 is a key initiator in response to infection, stress, and injury, and plays an important role in linking peripheral inflammation to central neuroinflammation. Studies have shown that, upon activation by external stimuli, TLR4 primarily signals through two downstream pathways. The first is the MyD88-dependent pathway, in which the adaptor protein MyD88 recruits kinases such as IRAK4, thereby activating I $\kappa$ B kinase (IKK) and MAPKs, leading to nuclear translocation of the transcription factors NF- $\kappa$ B and AP-1 and the rapid induction of pro-inflammatory cytokines [42, 43]. The second is the TRIF-dependent pathway, in which the adaptor protein TRIF activates TBK1, induces IRF3 phosphorylation, and promotes the production of type I interferons [44, 45]. In the present study, we found that TLR4 was the only Toll-like receptor broadly expressed in the astrocyte. Furthermore, STRING protein-protein interaction network analysis revealed that TLR4- and IRF7-related signaling events were primarily concentrated in the MyD88-independent TRIF/TRAM pathway. We speculate that *Pg* and its virulence, after being recognized by glial TLR4, activate IRF7 via the TRAM/TRIF signaling axis, thereby promote the release of IFN- $\beta$  and subsequently regulating the neuronal IFITM3-A $\beta$  pathway. Astrocytes exhibited the most pronounced changes during this process, further suggesting that they may play a central regulatory role in this proposed signaling cascade. However, the present study has not fully elucidated the specific regulatory mechanism by which TLR4 regulates IRF7 in glial cells, and this question warrants further investigation. Notably, pirinixic acid treatment significantly suppressed *TLR4* expression, further suggesting that PPAR- $\alpha$  agonists may regulate the IRF7-IFN- $\beta$ -IFITM3-A $\beta$  signaling pathway by inhibiting *Pg*-induced the activation of glial TLR4. These findings provide a new theoretical basis and potential therapeutic targets for early intervention in populations at risk for periodontitis-associated AD

In summary, this study delineates a novel periodontitis-activated IRF7-IFN- $\beta$ -IFITM3-A $\beta$  axis in glial cells that mechanistically links oral infection to AD-like pathology. By identifying PPAR- $\alpha$  agonism as a strategy to disrupt this pathway, our work provides a translational framework for early intervention in individuals with periodontitis at risk of cognitive decline.

## Conclusions

*Pg* activates glial cells through TLR4 recognition and drives periodontitis-associated AD-like pathology via the IRF7-IFN- $\beta$ -IFITM3 pathway, a process that is effectively blocked by pirinixic acid. These findings identify the glial IRF7-IFN- $\beta$ -IFITM3 axis as a central mechanistic link between periodontitis and AD pathogenesis.

## Abbreviations

A $\beta$ :  $\beta$ -amyloid

AD: Alzheimer's disease

APP: Amyloid precursor protein

BBB: Blood-brain barrier

CTD: Comparative Toxicogenomics Database

DEGs: Differentially expressed genes

FDR, False discovery rate

GEO: Gene Expression Omnibus

H&E: Hematoxylin and Eosin

IHC: Immunohistochemical

IFN: Interferon

IFITM3: Interferon-induced transmembrane protein 3

IKK: I $\kappa$ B kinase

IRF: Interferon regulatory factor

ISGs: Interferon (IFN)-stimulated genes

LPS: Lpopolysaccharide

NORT: Novel Object Recognition Test

PFA: Paraformaldehyde

*Pg*: Porphyromonas gingivalis

PHG: Parahippocampal gyrus

Pir: Pirinixic acid

PPAR- $\alpha$ : Peroxisome proliferator-activated receptor  $\alpha$

PRR: Pattern recognition receptor

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

SCP: Single-Cell Portal

Syn: Synapse

SnRNA-seq: Single-nucleus RNA sequencing

TICAM1: TIR domain-containing adaptor molecule

TLR4: Toll-like receptor 4

## Declarations

### **Ethics approval statement**

The animal care and experimental protocols were approved by the Animal Care and Use Committee of Dalian Medical University (No. AEE23140).

### **Clinical trial number**

Not applicable.

### **Consent for publication**

Not applicable

### **Availability of data and materials**

All data supporting the findings of this study are available within the paper and its Supplementary Information.

### **Competing interests**

The authors declare that they have no competing interests.

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## Authors' contributions

**FW** and **JJL**: Conceptualization, Methodology, Writing-original draft. **LLL** and **XC**: Investigation, Animal experiments. **JZ** and **YZY**: Investigation, Cell experiments. **YNJ**, **ASZ** and **MZL**: Formal analysis, Data curation. All authors read and approved the final manuscript.

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## Figures

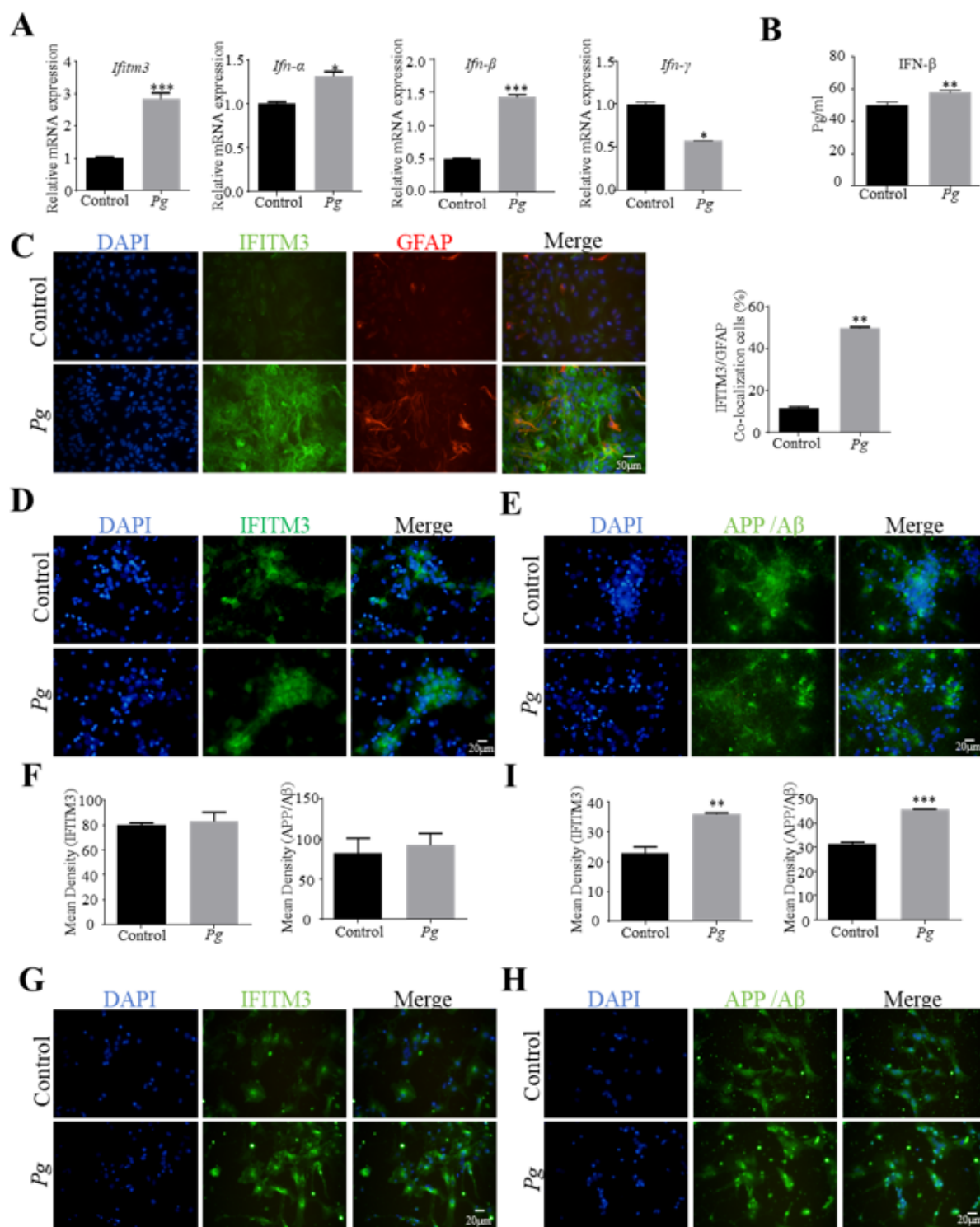


Figure 1

***Pg*-activated glial cells secrete IFN- $\beta$  and upregulate the neuronal IFITM3-A $\beta$  axis.** (A) mRNA levels of *Ifitm3*, *Ifn- $\alpha$* , *Ifn- $\beta$* , and *Ifn- $\gamma$*  in glial cells treated with or without *Pg*, measured by RT-qPCR. (B) Protein levels of IFN- $\beta$  in the supernatant of control and *Pg*-treated glial cells, assessed by ELISA. (C) Representative immunofluorescence images showing GFAP (red) and IFITM3 (green) in glial cells under different treatment conditions. Scale bar = 50 $\mu$ m. (D-F) Representative immunofluorescence images and

quantitative analysis of IFITM3 and APP/A $\beta$  expression in neurons directly treated with *Pg* or control. Scale bar = 20  $\mu$ m. (G–I) Immunofluorescence images and quantification of IFITM3 and APP/A $\beta$  expression in neurons treated with conditioned medium from control or *Pg*-stimulated glial cells. Scale bar = 20  $\mu$ m. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

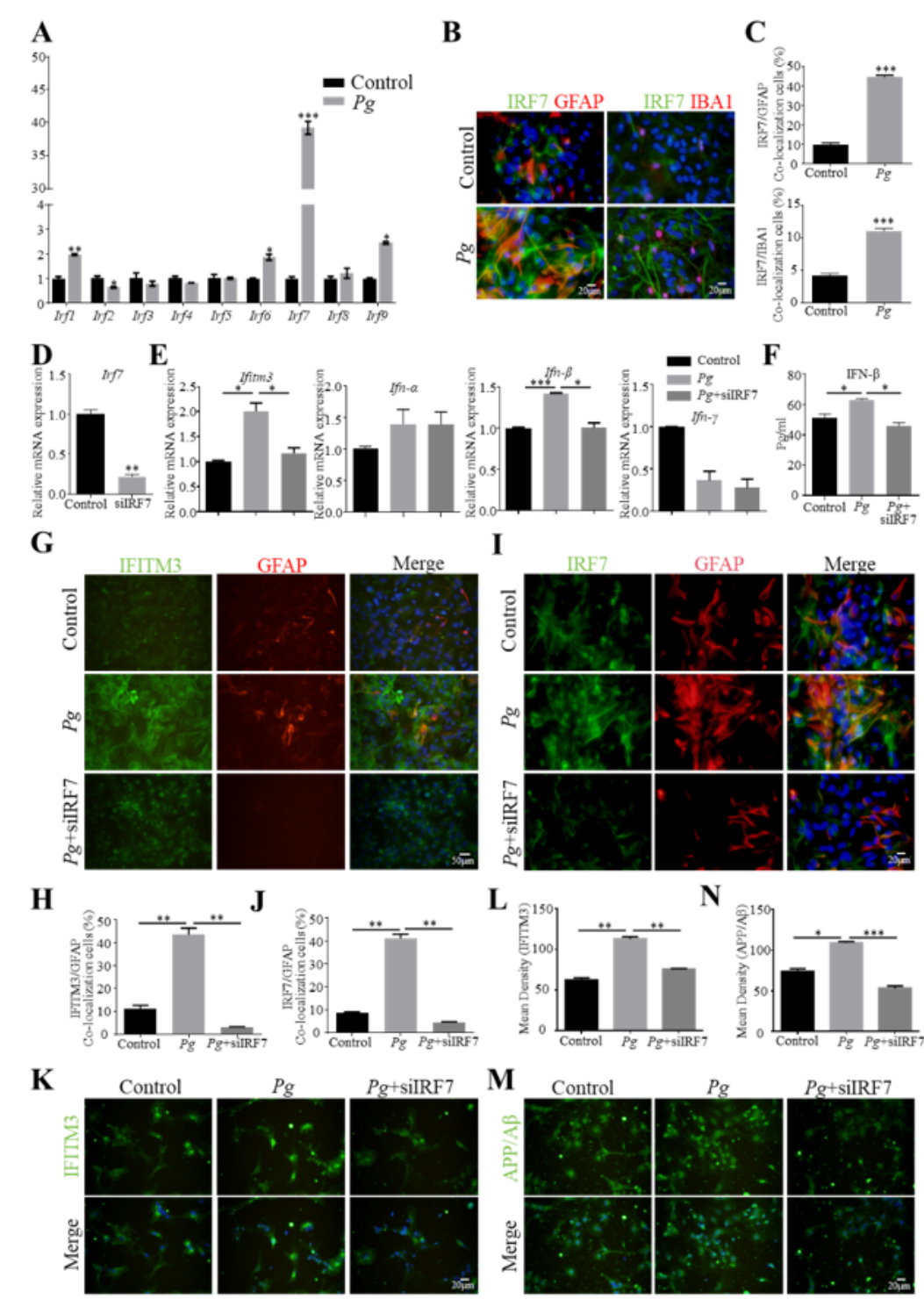
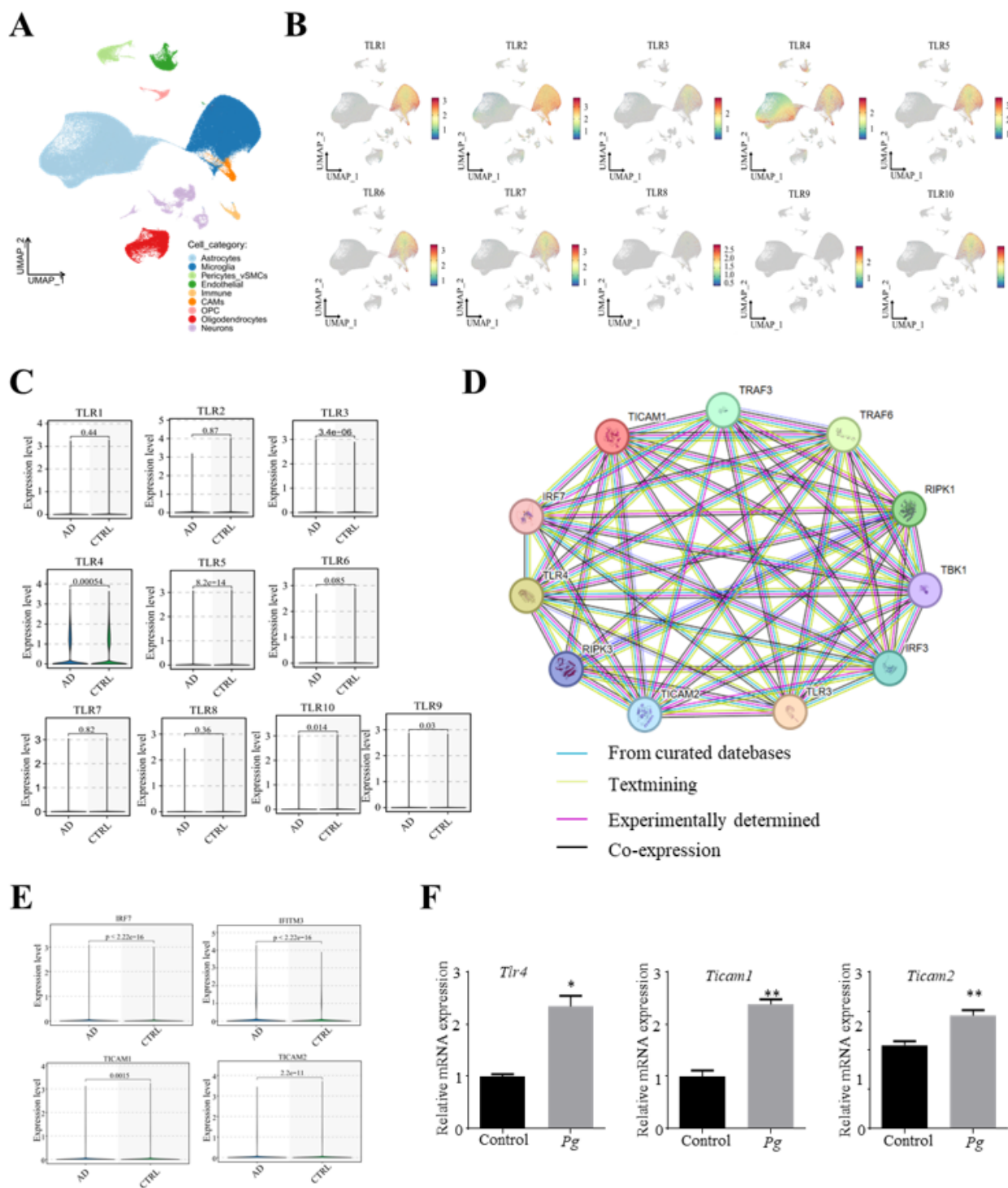


Figure 2

**IRF7 upregulated by *Pg* regulates IFN- $\beta$  production in glial cells.** (A) mRNA levels of *Irf1-Irf9* in glial cells treated with or without *Pg*, determined by RT-qPCR. (B, C) Representative immunofluorescence images and quantitative analysis showing co-localization of IRF7 (green) with GFAP (red, astrocyte marker) and with IBA1 (red, microglial marker). Scale bar = 20  $\mu$ m. (D) RT-qPCR detection of changes in *Irf7* gene levels in glial cells of the siIRF7 treatment group. (E) *Ifitm3*, *Ifn- $\alpha$* , *Ifn- $\beta$* , and *Ifn- $\gamma$*  mRNA levels in glial cells from the indicated groups, measured by RT-qPCR. (F) IFN- $\beta$  protein levels in the supernatant of glial cells from the indicated groups, assessed by ELISA. (G, H) Representative immunofluorescence images and quantification of GFAP (red) and IFITM3 (green) in glial cells under the indicated treatments. Scale bar = 50  $\mu$ m. (I, J) Immunofluorescence images and quantitative analysis of GFAP (red) and IRF7 (green) in glial cells from the indicated groups. Scale bar = 20  $\mu$ m. (K, L) Immunofluorescence staining and quantification of IFITM3 expression in neurons treated with conditioned medium from glial cells of the indicated groups. Scale bar = 20  $\mu$ m. (M, N) Immunofluorescence staining and quantification of APP/A $\beta$  expression in neurons treated with conditioned medium from glial cells of the indicated groups. Scale bar = 20  $\mu$ m. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 3**

**Pg activates glial cells through TLR4 recognition.** (A) Single-cell transcriptomic data from the GEO dataset GSE260461, including samples from patients with AD and cognitively normal controls, were analyzed. UMAP visualization showing the clustering of major cell types in the brain, including astrocytes, microglia, neurons, oligodendrocytes, and immune cells. (B) UMAP plot showing the distribution of TLRs expression across brain cell clusters, with relatively high expression observed in glial

cells (microglia and astrocytes). (C) Expression profiles of TLR family members (TLR1-TLR10) in astrocytes between AD patients and healthy controls. (D) Protein-protein interaction network analysis of TLR4 signaling pathway components demonstrating the interaction among TLR4, TICAM1 (TRIF), TICAM2 (TRAM), and IRF7. (E) Box plots showing the expression levels of *IRF7*, *IFITM3*, *TICM1*, *TICM2* in patients with AD and cognitively normal controls. (F) mRNA levels of *Tlr4*, *Ticam1*, *Ticam2* in glial cells measured by RT-qPCR. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

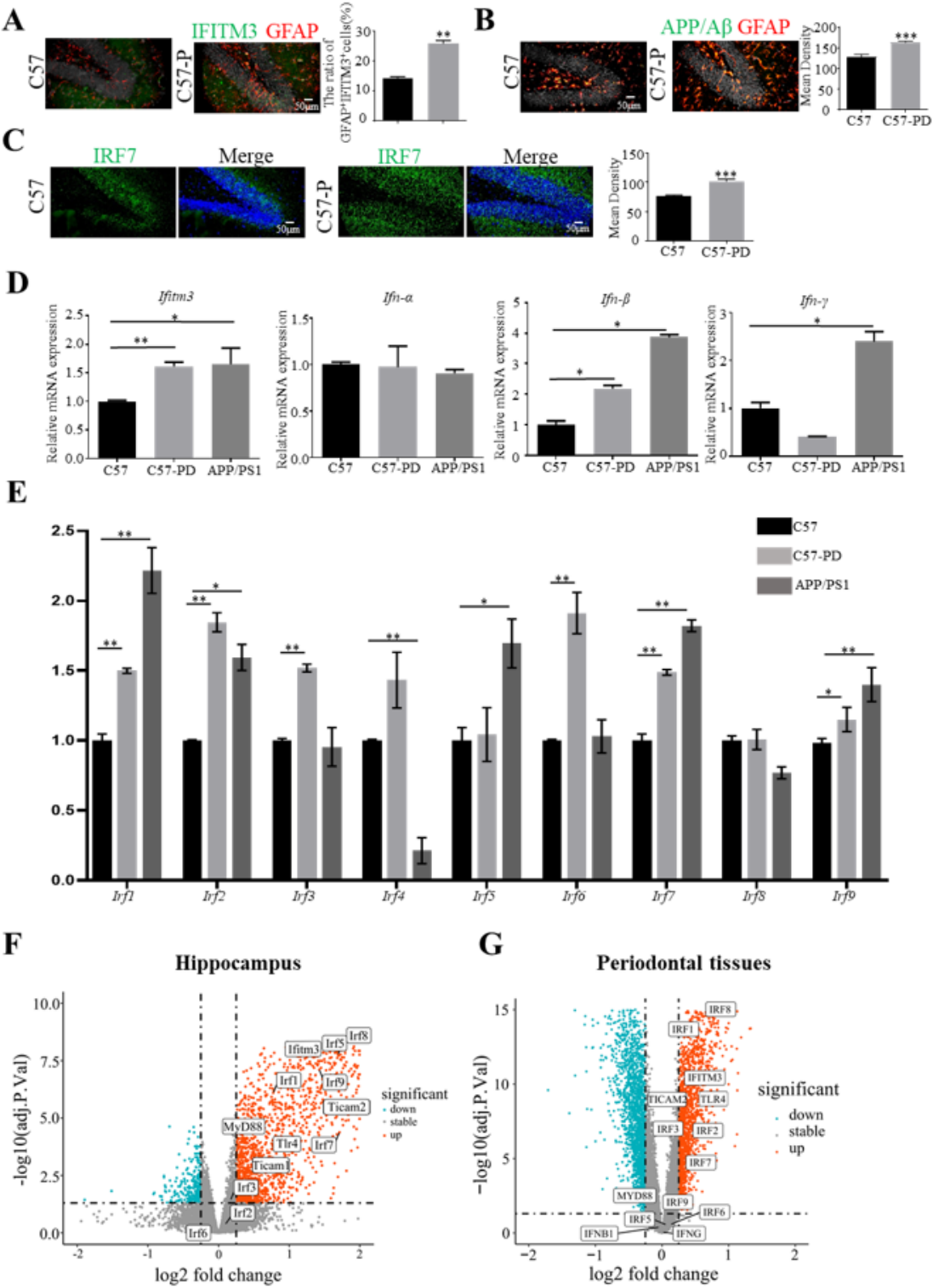
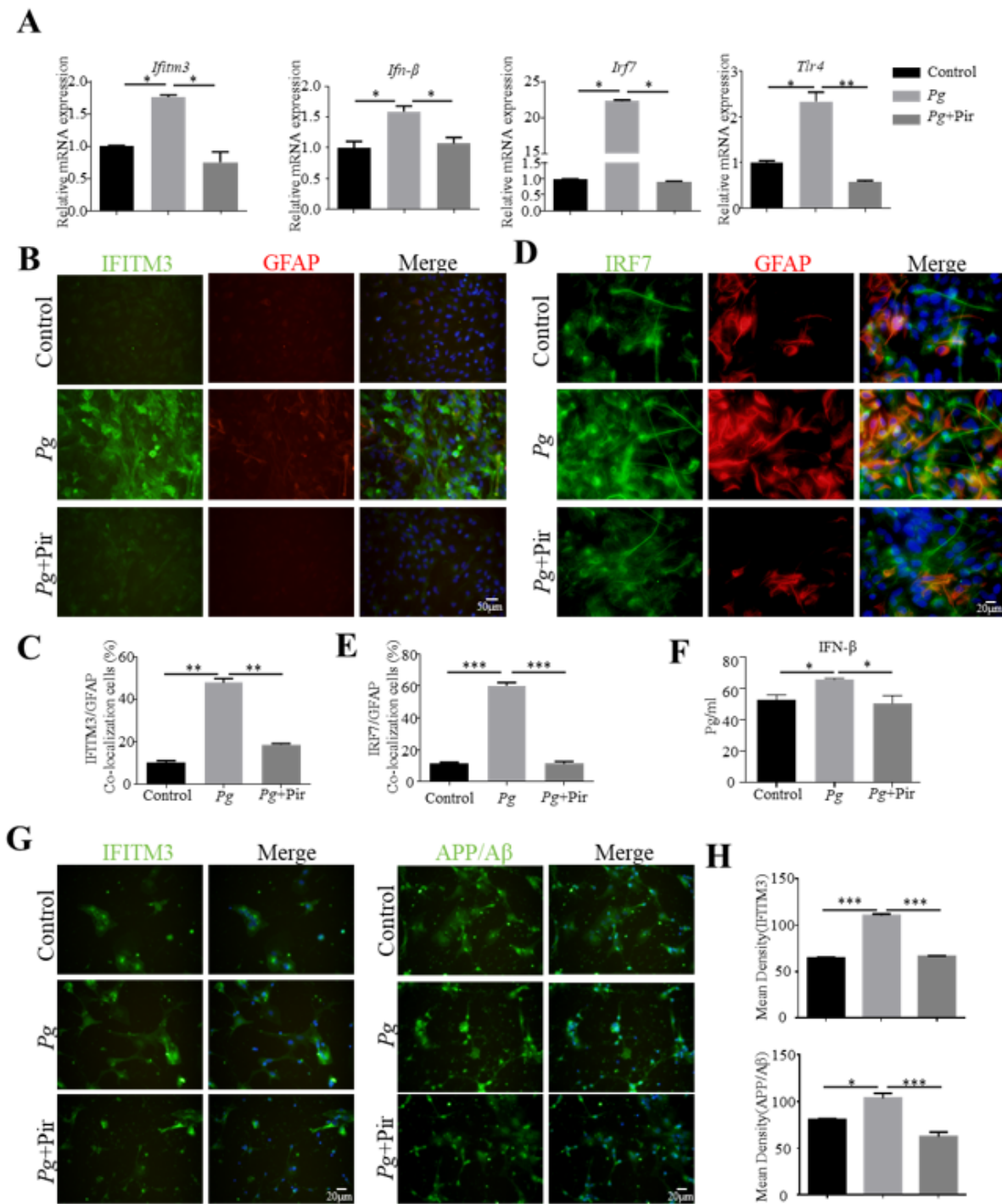


Figure 4

**The IRF7-IFN- $\beta$ -IFITM3 signaling axis is upregulated in the brains of periodontitis and AD model mice.**

(A) Representative immunofluorescence images and quantitative analysis showing co-expression of GFAP (red) and IFITM3 (green) in brain sections from C57BL/6J control (C57) and periodontitis (C57-P) mice. Scale bar = 50  $\mu$ m. (B) Immunofluorescence staining and quantification of GFAP (red) and APP/A $\beta$  (green) co-expression in brain sections from C57 and C57-P mice. Scale bar = 50  $\mu$ m. (C) Representative immunofluorescence images and quantitative analysis of IRF7 expression in the brains of C57 and C57-P mice. Scale bar = 50  $\mu$ m. (D) mRNA levels of *Ifitm3*, *Ifn- $\alpha$* , *Ifn- $\beta$* , and *Ifn- $\gamma$*  in the hippocampus of C57, C57-P, and APP/PS1 mice, determined by RT-qPCR. (E) mRNA levels of *Irf1-Irf9* in the hippocampus of C57, C57-P, and APP/PS1 mice, determined by RT-qPCR. (F) IFN-related gene expression levels are elevated in AD brains, as analyzed from a public RNA-seq dataset (GSE168137, 9 5xFAD model mice and 11 wild-type mice). (G) Compared with the normal group, the expression level of IFN-related genes in the periodontal tissues of the periodontitis group was increased as analyzed from a public RNA-seq dataset (GSE16134, 241 periodontitis patients and 69 healthy controls). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 5**

**Pirinixic acid attenuates *Pg*-induced IFITM3-A $\beta$  upregulation.** (A) mRNA levels of *Ifitm3*, *Ifn-β*, *Irf7*, and *Tlr4* in glial cells from Control, *Pg*, and *Pg+Pir* groups, determined by RT-qPCR. (B, C) Representative immunofluorescence images and quantitative analysis showing co-localization of GFAP (red) and IFITM3 (green) in glial cells under the indicated treatments. Scale bar = 50  $\mu$ m. (D, E) Immunofluorescence images and quantification of GFAP (red) and IRF7 (green) co-expression in glial

cells from the indicated groups. Scale bar = 20  $\mu$ m. (F) IFN- $\beta$  protein levels in the supernatant of glial cells from Control, *Pg*, and *Pg+Pir* groups, measured by ELISA. (G, H) Immunofluorescence staining and quantification of IFITM3 and APP/A $\beta$  expression in neurons treated with conditioned medium from glial cells of the indicated groups. Scale bar = 20  $\mu$ m. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

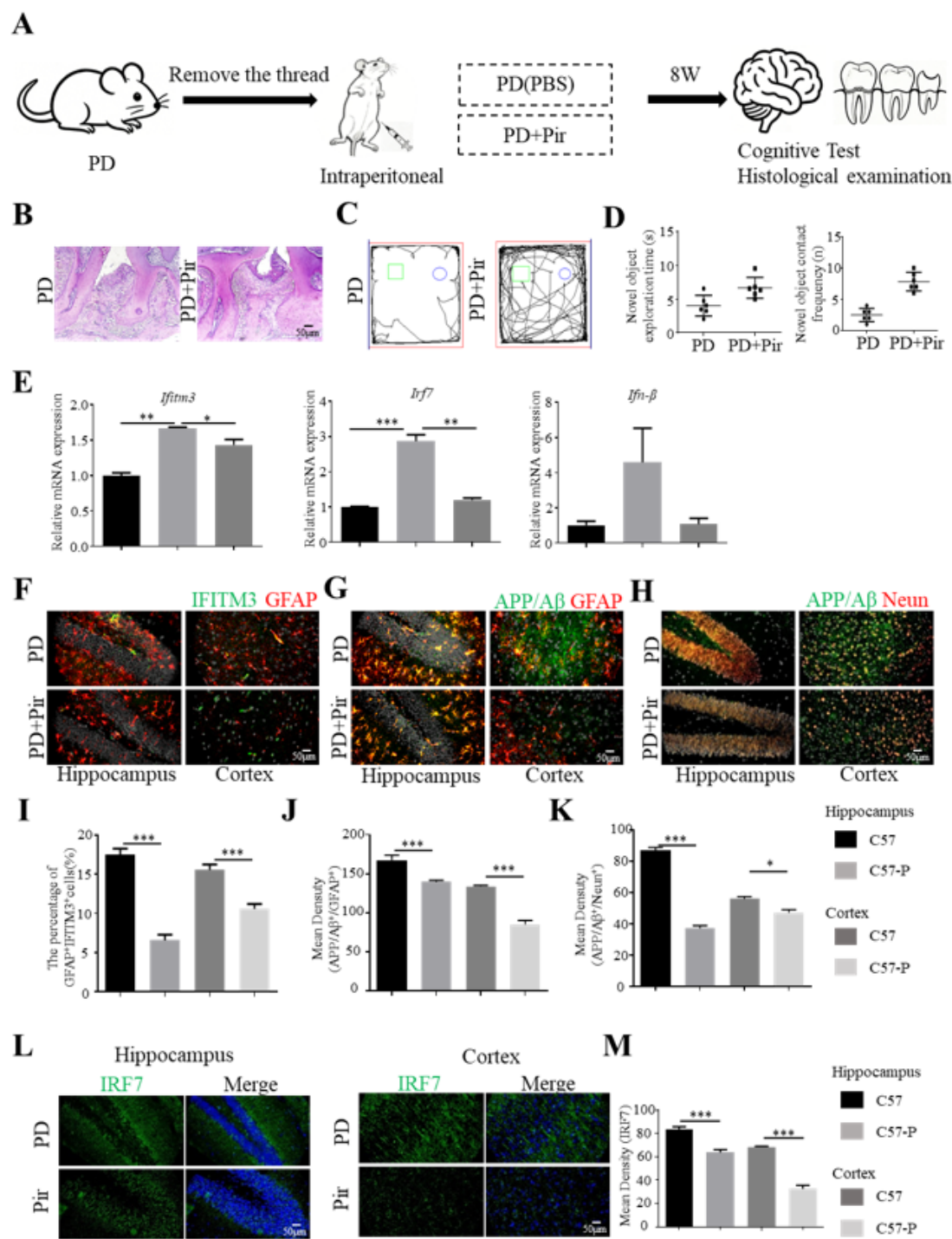


Figure 6

**Pirinixic acid alleviates periodontitis-induced neuroinflammation and cognitive decline in mice.** (A) Schematic diagram of the experimental timeline for pirinixic acid (Pir) treatment in mice with periodontitis. (B) Representative H&E-stained sections of maxillae from periodontitis (PD) and pirinixic acid-treated (Pir) mice. Scale bar = 50  $\mu$ m. (C, D) Representative movement tracks from the NORT and quantification of novel object exploration time and novel object contact frequency in PD and Pir groups. (E) mRNA levels of *Ifitm3*, *Irf7*, and *Ifn- $\beta$*  in the hippocampus of PD and Pir mice, determined by RT-qPCR. (F, I) Representative immunofluorescence images and quantitative analysis of IFITM3 (green) and GFAP (red) co-expression in brain sections of PD and Pir mice. Scale bar = 50  $\mu$ m. (G, J) Immunofluorescence staining and quantification of GFAP (red) and APP/A $\beta$  (green) expression in brain sections of PD and Pir mice. Scale bar = 50  $\mu$ m. (H, K) Representative immunofluorescence images and quantitative analysis of APP/A $\beta$  (green) and Neun (red) co-expression in brain sections of PD and Pir mice. Scale bar = 50  $\mu$ m. (L, M) Immunofluorescence staining and quantification of IRF7 expression in brain sections of PD and Pir mice. Scale bar = 50  $\mu$ m. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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