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JAK3 restrains inflammatory responses and protects against periodontal disease through Wnt3a signaling

Lanhai Lü[1,2,ξ], **Lan Yakoumatos**[1,ξ], **Junling Ren**[1], **Xiaoxian Duan**[3], **Huaxin Zhou**[4], **Zhen Gu**[1], **Muddasir Mohammed**[1], **Silvia M. Uriarte**[1,5], **Shuang Liang**[1], **David A. Scott**[1], **Richard J. Lamont**[1,*], **Huizhi Wang**[1,*,§]

[1]Department of Oral Immunology and Infectious Diseases, University of Louisville School of Dentistry, Louisville, KY 40202, USA.

[2]Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40202, USA.

[3]Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40202, USA.

[4]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, USA.

[5]Department of Medicine, School of Medicine, University of Louisville, Louisville, KY 40202, USA.

Abstract

Homeostasis between pro- and anti- inflammatory responses induced by bacteria is critical for the maintenance of health. In the oral cavity, proinflammatory mechanisms induced by pathogenic bacteria are well-established; however, the anti-inflammatory responses that act to restrain innate responses remain poorly characterized. Here, we demonstrate that infection with the periodontal pathogen *P. gingivalis* enhances the activity of JAK3 in innate immune cells, and subsequently phospho-inactivates Nedd4-2, a ubiquitin E3 ligase. In turn, Wnt3 ubiquitination is decreased, while total protein levels are enhanced, leading to a reduction in proinflammatory cytokine levels. In contrast, JAK3 inhibition or Wnt3a robustly enhances NF-κB activity and the production of proinflammatory cytokines in *P. gingivalis*-stimulated innate immune cells. Moreover, using gain- and loss-of-function approaches, we demonstrate that downstream molecules of Wnt3a signaling, including Dvl3 and β-catenin, are responsible for the negative regulatory role of Wnt3a. In addition, using an *in vivo P. gingivalis*-mediated periodontal disease model, we show that JAK3 inhibition enhances infiltration of inflammatory cells, reduces expression of Wnt3a and Dvl3 in *P.*

[*]**Correspondence: Huizhi Wang**, Ph.D.; M.D.; Associate Professor, VCU Philips Institute for Oral Health Research, Oral and Craniofacial Molecular Biology, School of Dentistry, Virginia Commonwealth University, Perkinson Bldg, Room 4152, 521 N. 11th St. Richmond, Virginia 23298-0566, wangh3@vcu.edu, Lab: (804) 628-6298, Office: (804) 628-6386 Fax: (804) 828-0150; **Richard J. Lamont** Oral Immunology and Infectious Diseases, University of Louisville, School of Dentistry, 501 S Preston St, Louisville, KY 40292, rich.lamont@louisville.edu, Office: (502) 852 2112, Fax: (502) 852 6394.

[§]Current address: Department of Oral and Craniofacial Molecular Biology, VCU School of Dentistry, Virginia Commonwealth University, 521 N. 11th St. Richmond, VA 23298-0566

[ξ]These authors contributed equally to this study.

Authors' Contribution

L. Lu, L. Yakoumatos, J. Ren, X. Duan, H. Zhou, Z. Gu, and M. Mohammed performed research; H. Wang and R. Lamont designed research; R. Lamont, S. Uriarte, and D. Scott contributed new reagents and analytic tools; H. Wang, L. Lu, and S. Liang analyzed data; H. Wang wrote paper and R. Lamont proofread it. S. Liang, S. Uriarte, and D. Scott contributed to the manuscript review. All authors read and approved the final manuscript.

gingivalis-infected gingival tissues, and increases disease severity. Together, our results reveal a new anti-inflammatory role for JAK3 in innate immune cells and show that the underlying signaling pathway involves Nedd4-2-mediated Wnt3a ubiquitination.

Keywords

JAK3; Inflammation; Ubiquitination; *P. gingivalis*; Wnt3

Introduction

Although an appropriate level of inflammation is beneficial in multiple physiological processes, excessive or prolonged bacteria-induced innate responses are a major and direct basis of pathology in a number of diseases (1, 2). In the oral cavity, periodontitis represents a highly prevalent disease involving inflammatory-driven tissue destruction induced upon bacterial challenge (3, 4). *Porphyromonas gingivalis*, a Gram-negative anaerobe, is a major pathogen in the initiation and progression of periodontitis. *P. gingivalis* can induce a destructive inflammatory response through a wide array of virulence factors such as lipopolysaccharide, fimbriae, capsule, and various proteases. In addition, gingipain proteases, secreted through the Type IX Secretion system can directly impinge upon tissue integrity. Moreover, colonization of *P. gingivalis*, even in a low abundance, facilitates the outgrowth of the surrounding microbial community, promotes dysbiosis and thus leads to dysregulation of inflammatory responses (1, 3, 5). Specifically, *P. gingivalis* colonization leads to the overproduction of proinflammatory cytokines (TNFα, IL-6, IL-12, and IL-1β), infiltration of inflammatory cells into gingival tissue (M1 macrophages, neutrophils, and Th17 cells), and the release of multiple enzymes, particularly proteases, that ultimately lead to soft tissue destruction and alveolar bone loss (1, 5). Homeostasis is dependent on a number of anti-inflammatory cytokines (IL-10, IL-4, IL-1Ra, and TGF-β) and anti-inflammatory cells (M2 macrophages and T-regs) that are activated to protect against the negative outcomes of excessive inflammation (6). Thus, the ratio between pro- and anti-inflammatory activity, to a large extent, dictates health status. However, the mechanisms that control the anti-inflammatory responses to *P. gingivalis* remain unclear. In this study we use *P. gingivalis*-induce inflammation as a model to elucidate key anti-inflammatory mechanisms which may also be pertinent to multiple infectious and non-infectious inflammatory conditions.

Janus kinases (JAKs) are cytoplasmic tyrosine kinases, which include four members designated JAK1, JAK2, JAK3, and Tyk2. JAKs are key regulators of immune homeostasis. Through binding to the intracellular moieties of type I and type II receptors, JAKs form homo- or hetero- dimers and phosphorylate each other, resulting in the phosphorylation of tyrosine residues on the intracellular domain of the receptors (7). These phosphorylated residues serve as docking sites for subsequent STAT transcription factors. Genetic studies have shown that deficiencies in either JAK1/2 or Tyk2 lead to severe combined immune deficiency or increased susceptibility to infection (8). Although JAK-mediated signaling is critical for the development of immune-mediated inflammatory disorders, studies have reported contradictory roles between the innate and adaptive immune systems for the

different JAK isoforms, especially for JAK3 (9-12). JAK3 inhibition has been widely investigated and is regarded as a promising therapeutic target to reduce excessive adaptive immune responses. Moreover, the JAK3 inhibitor tofacitinib has been applied clinically to treat multiple diseases, including rheumatoid arthritis, psoriasis, and other inflammatory disorders (9, 13, 14). However, increasing evidence suggests that JAK3 can play an opposing role in innate immune responses. Inhibition of JAK3 increases proinflammatory cytokine levels and exacerbates the progression of inflammatory responses in different models (10, 11). In addition, inhibition of JAK3 has also been shown to increase the activity of Th17 cells and thus aggravate the progression of inflammation (15). We previously demonstrated that the activation of JAK3 affects several downstream signaling pathways, such as PI3K, GSK3β, and STATs, through which JAK3 is involved in the innate immune response (11). However, how JAK3 affects these signaling pathways and whether other signaling pathways are also involved remains unknown, especially in response to *P. gingivalis* challenge.

Wnt signaling was originally considered a regulator of cell proliferation and differentiation in epithelial cells. Both the amount and localization of Wnt proteins are associated with the progression of multiple malignant diseases, including oral cancer (16, 17). Recent studies have demonstrated that Wnt3a signaling can also promote or suppress bacterial-mediated inflammation through distinct mechanisms (18-20). Moreover, the downstream responders of canonical Wnt3a signaling, GSK3β-β-catenin, can suppress bacterial-induced inflammation through the binding of β-catenin to NF-κB (21-23). We and others have demonstrated that Wnt3a canonical signaling restrains TLR4-mediated inflammation in innate immune cells (24-27). Further, the expression of Wnt3a and its suppressor, DKK1, has been reported to be associated with the progression of inflammation and is also involved in periodontal disease (26, 28). *P. gingivalis* can induce an inflammatory response through binding to different TLR receptors on the surface of immune cells (29, 30). However, the influence of *P. gingivalis*-Wnt3 signaling has yet to be investigated, although purified *P. gingivalis* LPS was recently reported to enhance the expression of Wnt3a (31).

Ubiquitination is an important posttranslational modification with functions that extend beyond its original role as a tag for protein degradation. The phosphorylation of E3 ubiquitin ligases is critical for their activity and controls the specificity of ubiquitination (29, 30). Crosstalk between ubiquitination and phosphorylation events has become a recurrent theme in cell signaling regulation (32). As with most other proteins, the downstream signaling molecules of Wnt3a-mediated canonical signaling, such as Dvl3 and β-catenin, are controlled by ubiquitination-mediated degradation (33, 34). Accumulating evidence has shown that *P. gingivalis* challenge leads to a series of changes in ubiquitin E3 ligase activity in different cells (29, 35). Although the influence of *P. gingivalis* infection on the phosphorylation of different kinases and downstream signaling pathway proteins has been widely investigated, the interactions between *P. gingivalis*-mediated phosphorylation and ubiquitination and its impact on the subsequent inflammatory responses remain unknown.

In this study, we utilized primary innate immune cells and a *P. gingivalis* infection-induced mouse periodontal bone loss model to investigate the anti-inflammatory function of JAK3 signaling and to elucidate the interaction between JAK3 and Wnt3a signaling. Importantly, we are the first to reveal that JAK3 can function as an inflammatory rheostat by modifying

the ubiquitination-mediated Wnt3a-β-catenin signaling pathway upon challenge with *P. gingivalis in vitro* and *in vivo*.

Materials and Methods

Mice, cells, and reagents

Eight-week-old C57BL/ mice were from The Jackson Laboratory and housed in a specific pathogen-free facility at the University of Louisville. All animal protocols were approved by the University of Louisville Institutional Animal Care and Use Committee. Peripheral blood mononuclear cells (PBMCs) were obtained from the venous blood of healthy donors as per protocols approved by the University of Louisville, Institutional Review Board. Monocytes were isolated by negative selection using the human monocyte isolation kit II from Miltenyi Biotec Inc. The purity of monocytes was routinely >90%, as determined by flow cytometry using a FITC-labeled anti-CD14 antibody. THP-1 and RAW 264.7 are human and murine leukemia cell lines, respectively, purchased from ATCC. Rabbit anti-JAK3 (D1H3; Cat. 8827), antibodies targeting phospho-JAK3 (Tyr980/981; D44E3; Cat. 5031), NEDD4L (Cat. 4013), Wnt3a (C64F2; Cat. 2721), Dvl3 (Cat. 3218), phospho-GSK3β (Ser9; D85E12; Cat. 5558), β-Catenin (Cat. 9581), phospho-β-Catenin (Ser33/37/Thr41; Cat. 9561), phospho-NF-κB p65 (Ser536; 93H1; Cat. 3033), K48 linkage-specific polyubiquitin (D9D5; Cat. 8081), K63 linkage-specific polyubiquitin (D7A11; Cat. 5621), ubiquitin (Cat. 3933), GAPDH (D16H11), and a horseradish peroxidase (HRP)-linked goat anti-rabbit IgG antibody (7074) were purchased from Cell Signaling Technology, Inc. Anti-NEDD4-2 (Ser448; Cat. ab168349), anti-Wnt3a (Cat. ab28472), anti-IL-6 (Cat. ab208113), anti-TNFα (Cat. ab215188), and anti-IL-12P40 (Cat. ab77373) antibodies were from Abcam. Rabbit anti-JAK3 (Cat. 07-1487) and rabbit anti-Wnt3a (Cat. 09-162) antibodies were purchased from EMD Millipore. Goat anti-phospho-JAK3 (Tyr980; Cat. sc-16567) and rabbit anti-JAK3 (C-21; Cat. sc-513) antibodies were purchased from Santa Cruz Biotechnology. The rat IgG2A isotype control (Cat. MAB006) was purchased from R&D Systems. Fetal bovine serum (Cat. S11150H) was purchased from Atlanta Biologicals. P40RPMI 1640 medium, L-glutamine, Lipofectamine® RNAiMAX Transfection Reagent, radioimmunoprecipitation assay (RIPA) Lysis and Extraction Buffer, Micro BCA™ Protein Assay kits, all Western Blot buffers, SuperSignal™ West Pico Chemiluminescent Substrate, SeeBlue® Plus2 Prestained Protein Standard, SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Pierce® Crosslink Immunoprecipitation kits, and MagicMark™ XP Western Protein Standard were from Thermo Fisher Scientific. MACS® LS Columns (130-042-401) were from Miltenyi Biotec. The proteasome inhibitor MG132 (also known as carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal; Z-LLL-CHO) (S2619) was purchased from Selleckchem. Tofacitinib citrate (T-1377, 540737-29-9), Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK; 90182), Phosphatase Inhibitor Cocktail 3 (P0044), and fetal bovine serum (F-4135-500 ml) were purchased from Sigma. Small interfering RNAs (siRNAs) against JAK3, NEDD4L, Wnt3a, GSK3β, and β-catenin were purchased from Dharmacon or Santa Cruz Biotechnology, Inc. All plasmids including pcDNA3-S33Y β-catenin (Cat.19286) with Flag tag, pcDNA3 Flag HA (Cat. 10792), which was constructed with HA and Flag tags, HA GSK3β S9A pcDNA3 (Cat. 14753), and pDONR223-JAK3 (Cat. 23944) were from Addgene. Primary Cell Nucleofector™ kits for the 4D-Nucleofector™ X Unit

(V4XP-3024) were purchased from Lonza. Human IL-12/IL-23 (p40) ELISA MAX™ Deluxe, human IL-6, and human TNFα cytokine ELISA kits were purchased from Biolegend or eBioscience. RNeasy Mini (74104) and QuantiTect SYBR Green PCR (204143) kits were from QIAGEN. The SuperScript™ First-Strand Synthesis System for RT-PCR (11904-018) was purchased from Invitrogen.

Bacteria and cell culture

Porphyromonas gingivalis 33277 was cultured anaerobically in trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml). *S. sanguinis* SK36 (from Dr. Kitten's lab) and *S. gordonii* DL1 were grown at 37°C in Anoxomat jars (Spiral Biotech) under microaerobic conditions (7% H_2, 7% CO_2, 80% N_2, and 6% O_2) in brain-heart infusion (BHI; Bacto, Sparks, MD) broth. PBMCs were obtained from healthy donors according to protocols approved by the University of Louisville Institutional Review Board. Monocytes were isolated by negative selection using a Human Monocyte Isolation Kit II from Miltenyi Biotec (130-091-153). For bacterial treatment, monocytes isolated from PBMCs were directly seeded in either 96- or 6-well plates. RAW 264.7 (ATCC® TIB-71™) cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum and penicillin-streptomycin (100 U/mL). Human monocytic leukemia cells (THP-1) were purchased from ATCC (ATCC® TIB-202™) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin (100 U/mL). Cells were cultured at a temperature of 37°C in a humidified growth chamber under 5% CO_2.

ELISA, Western blot, and siRNA and plasmid transfection

For ELISAs, 2,000 cells were plated per well in a 96-well plate. After overnight culture, the cells were treated with or without 10 nM T-1377 for 4 h. Fifty nM TLCK-pretreated *P. gingivalis* was added to the cells according to the desired MOI. After 24 h, the supernatant was collected and stored at −20° C for the measurement of cytokine levels by ELISA (eBioscience). For Western blot assays, the cells in six-well plates were treated with *P. gingivalis* for the desired time points and lysed with RIPA buffer (Sigma) containing phosphatase and protease inhibitors. Protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Thermo). Samples were separated on NuPage Novex 4 to 12% Bis-Tris Polyacrylamide Gels (Invitrogen) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). For siRNA and plasmid transfections, human monocytes were electroporated using a 4D-Nucleofector™ System (Lonza) according to the manufacturer's protocols. Briefly, 4×10^6 purified monocytes were resuspended in 100 μl of Nucleofector Solution (P3 Primary Cell 4D-NucleofectorTM X Kit L, Lonza, V4XP-3024) containing 2 μg of siRNA duplexes or ectopic plasmids for each target. After electroporation, the cells were directly seeded in either 96- or 6-well plates. For RAW264.7 or THP-1 cells, the cells were transfected with siRNA duplexes using Lipofectamine® RNAiMAX Transfection Reagent. After 72 hours, cells were treated with *P. gingivalis* for 24 or 48 h. Supernatant was collected for cytokine level detection by ELISA (eBioscience). Cells were lysed in RIPA buffer containing phosphatase inhibitors for Western blot assays. For siRNA studies, the levels of total JAK3, Nedd4-2, Wnt3a, GSK3β and β-actin were assessed by Western blot on day 3 after transfection.

Immunoprecipitation

Human monocytes or RAW264.7 cells were pretreated with the proteasome inhibitor MG132 for 4 h before stimulation with *P. gingivalis*, following which the cells were collected and centrifuged. The cells were lysed in immunoprecipitation lysis/wash buffer containing the proteasome inhibitors and phosphatase inhibitors, which were maintained throughout the experiments. After incubation on ice for 30 min, the lysates were collected by centrifugation for 20 min at 12,000 × g at 4°C. The following procedures were performed according to the Pierce Coimmunoprecipitation (Co-IP) kit (26149, Thermo Scientific™) instructions. For the detection of JAK3 phosphorylation, the cell lysate was precleared by adding the control agarose resin. After incubation at 4°C for 1 h with gentle end-over-end mixing, the precleared lysate was collected by centrifugation at 1000 × g for 1 min. For immunoprecipitation reactions, the precleared supernatants were rotated with 2 μl of JAK3 antibody at 4°C overnight. The JAK3 immune complexes were captured by incubation for 2 h at 4°C with protein-A/G sepharose beads. After incubation, the beads were washed five times with cold immunoprecipitation lysis buffer containing protein inhibitors and eluted by boiling in 1× SDS sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.02% bromophenol blue, 10% glycerol). Samples were examined by Western blot assays with phospho-JAK3 (Tyr980/981) antibody or other antibodies at 4°C overnight. For wnt3a immunoprecipitation, 50mM N-ethylmaleimide (NEM) was added in the lysis buffer to preserve its ubiquitylation. The membranes were developed with HRP-conjugated goat anti-rabbit IgG (heavy plus light chains; KPL) or horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; heavy plus light chains), visualized by SuperSignal™ West Pico PLUS Chemiluminescent Substrate and imaged by an ImageQuant LAS 4000.

P. gingivalis-induced bone loss model and immunohistochemistry

The endogenous oral microbiota was suppressed in 10- to 12-week-old C57/B6/J mice by sulfamethoxazole (800 μg/ml) and trimethoprim (400 μg/ml) provided *ad libitum* in water for 5 days. The mice then received pure drinking water for 5 days. Alveolar bone loss was induced by oral infection with 1×10^9 CFU of *P. gingivalis* suspended in 100 μl of phosphate-buffered saline with 2% carboxymethylcellulose. Infections were performed six times at every other day. An experimental group was also intraperitoneally (IP) administered T-1377 (15 mg/kg) with the infection and every other day thereafter until euthanization. Sham-infected and vehicle control mice were also established. The mice were euthanized with CO_2 and cervical dislocation 42 days after the final infection. Maxillary gingiva from mice upper jaws were harvested with half used for RT-PCR assay, and the other half for Western blot assay. The fresh gingival tissues were immersed in RNAlater® (Ambion Cat. AM7020) or RIPA buffer with protease and phosphatase inhibitors (Millipore Sigma, Cat. P8340 and P0044) (1:100) and then stored at −20°C for further RT-PCR or Western blot assay, respectively. The lower jaws of the mice were fixed in 4% paraformaldehyde, decalcified in immunocal solution for 15 d and embedded in paraffin wax for immunohistochemistry assay. Alveolar bone loss was measured in millimeters at 14 predetermined points per mouse on the maxillary molars of defleshed maxillae as the distance from the CEJ to the ABC. Bone loss was visualized by methylene blue/eosin staining and quantified using a Nikon SMX 800 dissecting microscope (40×) fitted with a Boeckeler VIA-170K video image marker measurement system. The results were expressed

as the mean with S.D. Jawbones. The paraffin embedded tissue blocks were freshly cut into 4 μm mesiodistal sections for subsequent immunostaining with hematoxylin and eosin (H&E) to evaluate inflammatory cell infiltration, and with mouse Wnt3a or Dvl3 antibodies with visualization using 3,3'-diaminobenzidine (DAB). Images were captured using a laser-scanning confocal microscope (Olympus FV1000) under bright field illumination with a color CCD and 20× objective, and analyzed by counting positively stained cells using Image J software. At least 5 serial tissue sections from the same block were used to measure the positive area and 20 adjacent fields of view from the same anatomic area of each tissue section were selected to count the total number of inflammatory cells. The immune-histochemistry staining for all sections was scored by means of the semiquantitative Immunoreactivity Score (IRS) described by Remmele and Stegner (36) and modified by McCarty et al. (37). In this system, the percentage of positive cells in five gradations (no positive cells [0]; <10% positive cells [1]; 10-50% positive cells [2]; 51-80% positive cells [3]; >80% positive cells [4]) is multiplied by the staining intensity in four gradations (no staining [0]; mild staining [1]; moderate staining [2]; strong staining [3]). As a result, score values between 0 and 12 were obtained.

RT-PCR and NF-κB p65 nuclear-binding assay

Total RNA was isolated using a RNeasy Mini kit (Qiagen), and real-time RT-PCR was performed using an Applied Biosystems 7500 system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping control. The amount of each target mRNA expressed in a sample was analyzed in triplicate and normalized to the amount of internal normalization control transcripts. The relative fold increase was calculated according to the ΔΔCt method. Nuclear lysates were obtained from human monocytes using a Nuclear/Cytosolic Isolation kit (Active Motif), and analyzed for DNA-binding levels of NF-κB p65 using TransAM NF-κB p65 (Active Motif) according to the manufacturer's protocol.

Statistical analyses

The statistical significance of differences between groups was evaluated by the analysis of variance and the Tukey multiple comparison test using the InStat program (GraphPad). Differences between groups were considered significant at the level of $P \leq 0.05$.

Results

P. gingivalis challenge robustly enhances the activity of JAK3 in innate immune cells

The activation of JAK3 has been reported to dampen TLR-mediated innate immune responses and suppress intestinal inflammation and polymorphonuclear (PMN) leukocyte infiltration (11). Since both TLR2 and TLR4 are involved in *P. gingivalis*-induced inflammation (38-40), we initially sought to examine the influence of *P. gingivalis* infection on the activity of JAK3 in innate immune cells. In a dose-response experiment we found that a multiplicity of infection (MOI) of 10 of *P. gingivalis* induced the production of ample amounts of inflammatory cytokines without substantial loss of cell viability (Fig. 1A and B). Moreover, *P. gingivalis* infection substantially increased JAK3 phosphorylation in human monocytes, THP-1 cells and RAW264.7 cells (Fig. 1C to F). We confirmed these results

using immunoprecipitation, and found that *P. gingivalis* infection elevated the level of JAK3 phosphorylation in innate immune cells (Fig. 1G). Further evidence using enriched immunoprecipitates also showed that *P. gingivalis* robustly enhances the phosphorylation of JAK3 in human monocytes (Fig. 1H). Collectively, these findings establish that the interaction of *P. gingivalis* with innate cells can increase activity of JAK3.

P. gingivalis-activated JAK3 restrains the production of proinflammatory cytokines in innate immune cells

To examine whether JAK3 regulates *P. gingivalis*-induced inflammatory responses, we inhibited JAK3 with a specific inhibitor, T-1377, which at a concentration of 1 nM significantly enhanced the production of TNFα, IL-6, and IL-12P40 in human monocytes, THP-1 cells, and RAW264.7 cells (Fig. 2A to C). To determine whether the T-1377 indeed suppresses the activity of JAK3, we assessed the phosphorylation of STAT-5 and STAT-6, two prototypical substrates of JAK3, and found that their phosphorylation level was robustly elevated in *P. gingivalis*-stimulated human monocytes (Fig. 2D). However, inhibition of JAK3 substantially suppressed phosphorylation of STAT-5 and STAT-6 (Fig. 2D). These results demonstrate for the first time that *P. gingivalis* infection activates JAK3, which subsequently restrains the production of inflammatory cytokines in innate immune cells. To exclude the possibility of nonspecific effects of the pharmacologic JAK3 inhibitor, we used pre-validated siRNAs to knockdown JAK3 in human monocytes. As shown in Fig. 2E and F, JAK3 silencing by specific siRNA (Fig. 2E) resulted in a significant increase in the TNFα, IL-6, and IL-12P40 levels in *P. gingivalis*-stimulated human monocytes (Fig. 2F). Moreover, a plasmid encoding exogenous JAK3 (Fig. 2G) was used to exclude the possible off-target effects of the siRNA and confirm the regulatory function of JAK3. We found that compared with control human monocytes JAK3-overexpressing monocytes (Fig. 2G) had significantly reduced production of TNFα, IL-6, and IL-12P40 in response to *P. gingivalis*-stimulation (Fig. 2H). These results establish the anti-inflammatory properties of JAK3 in *P. gingivalis*-stimulated innate immune cells.

P. gingivalis enhances the activity of Wnt3a-β-catenin signaling through JAK3 in innate immune cells

Bacterial infection has been reported to enhance the expression of Wnt3a and the downstream protein β-catenin to suppress inflammatory responses (41, 42). *P. gingivalis* has also been shown to modulate the Wnt3 signaling pathway and the downstream GSK3β-β-catenin axis in a human hepatoma cell line and gingival epithelial cells (43, 44). Thus, we examined whether Wnt3a responds to *P. gingivalis* infection and affects the inflammatory response in immune cells. We found that *P. gingivalis* infection significantly increased the expression of Wnt3a and Dvl3 in human monocytes (Fig. 3A) and THP-1 cells (Fig. 3D). Interestingly, *P. gingivalis* infection or JAK3 inhibition didn't induce a significant change in the mRNA levels of Wnt3a and Dvl3 in human monocytes (Fig. 3C), indicating *P. gingivalis* challenge may not affect the *de novo* synthesis of Wnt3a and Dvl3. Moreover, the downstream components of the canonical Wnt3 signaling pathway also responded to *P. gingivalis* infection with a marked increase in GSK3β phosphorylation and accumulation of β-catenin in human monocytes and THP-1 cells (Fig. 3A, D). These results suggest that infection with *P. gingivalis* enhances the expression of Wnt3a and promotes canonical

Wnt3a signaling activity. To determine if the increase of JAK3-Wnt3-Dvl3 is specific for *P. gingivalis* stimulation, we also utilized *Streptococcus sanguinis* and *Streptococcus gordonii* to treat monocytes and no substantial alteration of p-JAK3, Wnt3a and Dvl3 expression was observed (Fig. 3I, J). Considering both *S. sanguinis* and *S. gordonii* are Gram-positive, LPS from Gram-negative bacteria could be the key for the enhancement of Wnt3-Dvl3 expression. This is substantiated by a very recent finding about TLR4-enhanced Wnt3a expression, without a requirement for whole bacteria (45). Since we have previously demonstrated that JAK3 restrains TLR-mediated inflammation through GSK3β (11), we sought to determine whether there are interactions between JAK3 and Wnt3 signaling in *P. gingivalis*-stimulated cells. We found that inhibition of JAK3 diminished the *P. gingivalis*-enhanced expression of Wnt3a, Dvl3, phosphorylated GSK3β, and β-catenin in human monocytes and THP-1 cells (Fig. 3A to D). Moreover, siRNA-mediated JAK3 silencing confirmed these results in *P. gingivalis*-stimulated human monocytes (Fig. 3F to H), strongly suggesting that *P. gingivalis*-enhanced Wnt3a-β-catenin signaling is through JAK3 in innate immune cells.

JAK3-mediated Wnt3a-β-catenin signaling suppresses production of inflammatory cytokines in *P. gingivalis*-stimulated innate immune cells

Since Wnt3a has been demonstrated to suppress TLR-mediated inflammation (20, 27, 45, 46) in monocytes or macrophages, we next examined whether Wnt3-mediated GSK3β-β-catenin signaling restrains *P. gingivalis*-induced inflammatory responses. We found that siRNA-mediated Wnt3a or Dvl3 silencing led to a significant increase in the production of inflammatory cytokines, including TNFα, IL-6, and IL-12P40, in *P. gingivalis*-stimulated monocytes (Fig. 4A to C). In contrast, the silencing of GSK3β by siRNA (Fig. 4D) or overexpression of ectopic β-catenin (Fig. 4D) significantly decreased TNFα, IL-6 and IL-12P40 levels in *P. gingivalis*-stimulated human monocytes (Fig. 4E, F). In addition, overexpression of a constitutively active GSK3β construct (S9A; Fig. 4G) significantly elevated the production of TNFα, IL-6 and IL-12p40 (Fig. 4H) and decreased the accumulation of β-catenin in *P. gingivalis*-stimulated cells (Fig. 4G). These results demonstrate that *P. gingivalis* infection leads to the activation of canonical Wnt3a signaling, which restrains the production of proinflammatory cytokines through the downstream GSK3β-β-catenin signaling in innate immune cells. On the other hand, the effects of JAK3 inhibition or Wnt3a/Dvl3 gene silencing on the levels of inflammatory cytokines were abrogated by the inhibition of GSK3β or overexpression of β-catenin in *P. gingivalis*-stimulated human monocytes (Fig. 4E to F). Taken together, these data suggest that JAK3-mediated inhibition of *P. gingivalis*-induced inflammation is dependent, at least in part, on the activity of canonical Wnt3a signaling in innate immune cells.

JAK3 controls the expression of Wnt3a by phospho-inactivating Nedd4-2 and enhancing Wnt3a ubiquitination

Our data show that the inhibition of JAK3 affects the expression of Wnt3a and Dvl3 at the early stage of *P. gingivalis* infection, indicating that *P. gingivalis*-activated JAK3 could interfere the stability rather than the *de novo* synthesis of Wnt3a. To examine whether the effect of *P. gingivalis* on Wnt3a expression involves ubiquitination, and to examine the role of JAK3 in this process, a proteasome inhibitor, MG132, was utilized to block the activity of

the ubiquitination proteasome pathway. We found that MG132 treatment abrogated the effect of both *P. gingivalis* infection and JAK3 inhibition on the expression of Wnt3a in human monocytes (Fig. 5A to C). These results suggest that Wnt3a is indeed regulated by ubiquitination proteasomes pathway, which is further confirmed by the enhanced Wnt3a in all MG132-treated monocytes upon the challenge of *P. gingivalis* (Fig. 5B). We next immunoprecipitated Wnt3a from *P. gingivalis*-stimulated human monocytes pretreated with MG132 and examined the amount of K48 ubiquitination on the immunoprecipitated Wnt3a. We found that *P. gingivalis* infection substantially reduced, whereas siRNA-mediated JAK3 silencing increased, the amount of K48 ubiquitination (Fig. 5D). These results demonstrate that JAK3 affects the ubiquitin-mediated degradation of Wnt3a in *P. gingivalis*-stimulated human monocytes. Considering the interaction between phosphorylation and ubiquitination, we next sought to examine whether JAK3 activation affects the ubiquitin E3 ligases in *P. gingivalis*-stimulated cells. We found that *P. gingivalis* infection enhances the phosphorylation of the ubiquitin E3 ligase Nedd4-2 (Fig. 5E and F) in human monocytes and THP-1 cells, which leads to its inactivation (47, 48). Additionally, chemical and/or siRNA mediated JAK3 inhibition decreased the phosphorylation of Nedd4-2 in *P. gingivalis*-stimulated human monocytes and THP-1 cells (Fig. 5E and F). Moreover, deficiency of JAK3 simultaneously reduced the phosphorylation of Nedd4-2 and the expression of Wnt3a, indicating decrease of Nedd4-2 phosphorylation enhances its activity and thus promotes the degradation of Wnt3a. To confirm the effect of Nedd4-2 in *P. gingivalis*-stimulated innate immune cells, we utilized siRNA-mediated silencing of Nedd4-2 (Fig. 5G) and found that Nedd4-2 deficiency significantly reduced the production of inflammatory cytokines in *P. gingivalis*-stimulated human monocytes (Fig. 5H). Taken together, these results demonstrate that *P. gingivalis* infection activates JAK3 and subsequently enhances Wnt3a signaling through the phospho-inactivation of Nedd4-2 and consequent decrease in the degradation of Wnt3a in innate immune cells.

Inhibition of JAK3 or Wnt3 signaling increases *P. gingivalis*-induced NF-κB activity

Multiple inflammatory signaling pathways, such as NF-κB and MAPK, and a number of transcription factors are involved in the production of inflammatory cytokines (49, 50). Thus, we examined the nature of the transcription factors targeted by JAK3-mediated Wnt3a signaling in *P. gingivalis*-stimulated cells. We found that compared to controls, JAK3 pharmacological inhibition (Fig. 6A) or siRNA-mediated silencing of *jak3, wnt3*, or *dvl3* (Fig. 5B to D) led to a significant increase in NF-κB activity, represented by the phosphorylation of NF-κB p65 at serine 536 (Fig. 6A to D) in *P. gingivalis*-stimulated human monocytes. We next utilized the nuclear lysates to examine the influence of JAK3, Wnt3a and Dvl3 on the DNA-binding activity of NF-κB. As shown in Fig. 6E and F, JAK3 inhibition or silencing of *jak3, wnt3*, or *dvl3* by siRNA led to a significant increase in NF-κB activity in *P. gingivalis*-stimulated cells, indicating that JAK3-mediated Wnt3a signaling exerts anti-inflammatory effects by modifying the activity of NF-κB.

JAK3-Wnt3a pathway restrains inflammation in a *P. gingivalis*-induced bone loss model

P. gingivalis-induced inflammatory responses play a key role in the initiation and development of periodontitis (51). Since we found that JAK3 inhibition increases TNFα, IL-6, and IL-12P40 expression in *P. gingivalis*-stimulated human monocytes by reducing the

expression of Wnt3a and Dvl3, we next investigated whether JAK3 inhibition attenuates the activity of Wnt3 signaling and exacerbates the severity of periodontal bone loss using a mouse model of *P. gingivalis*-induced disease (52) (Fig. 7A). We first utilized quantitative nested PCR to test the efficiency of *P. gingivalis* infection, represented by the amount of DNA recovered from the oral cavity. We found that all mice infected with *P. gingivalis* were successfully colonized (Fig. 7B). We next examined TNFα, IL-6, and IL-12P40 in the gingival tissues and found that the expression of IL-6 was significantly enhanced in JAK3 inhibitor-treated *P. gingivalis*-infected mice compared to mice infected with *P. gingivalis* only, but no substantial differences were observed in the messenger RNA and protein levels of TNFα and IL-12P40 (Fig. 7C and E). This discrepancy between *in vitro* and *in vivo* response to JAK3 inhibition may be due to the phase of inflammation, differential dynamics of cytokines synthesis, variant resistance to *P. gingivalis*-induced tolerance, mutual regulation of cytokines, and importantly, the different components of samples, indicating that complicated regulatory mechanisms are involved in the progression of chronic inflammation. Because overwhelming neutrophil and macrophage accumulation has been demonstrated to significantly contribute to the development of periodontitis (53), we next examined whether JAK3 inhibition affects inflammatory cell infiltration by staining tissue sections with hematoxylin and eosin (H&E; Fig. 8A to C and I to K). We found that compared to no infection, *P. gingivalis* infection increased the infiltration of inflammatory cells in mice (Fig. 8H and P). Furthermore, the number of inflammatory cells in the gingival tissue of JAK3 inhibitor-treated mice was significantly increased compared to that in mice without JAK3 inhibitor treatment (Fig. 8H and P). In addition, *P. gingivalis* infection induced significant bone loss, and JAK3 inhibition aggravated the severity of the bone loss as determined through the measurement of the cementoenamel junction (CEJ)- alveolar bone crest (ABC) distance (Fig. 7F and G). Additionally, we employed an Immunoreactivity Score (IRS) system to evaluate the expression of Wnt3a and Dvl3 in murine gingival tissue. We found that *P. gingivalis* infection led to an increase in Wnt3a (Fig. 8A to F) and Dvl3 (Fig. 8I to N) expression (Fig. 8), and JAK3 inhibitor treatment significantly reduced the expression of these proteins in the gingival tissues (Fig. 8). However, there is no significant changes in the mRNA levels of Wnt3a and Dvl3 (Fig. 7D), indicating *P. gingivalis* infection or JAK3 inhibition may not affect *de novo* synthesis of Wnt3/Dvl3, which is consistent with the effects of the JAK3 inhibitor on Wnt3 signaling in *in vitro*. These results demonstrate that inhibition of JAK3 promotes *P. gingivalis*-induced inflammatory responses and aggravates periodontal bone loss in a mouse model, indicating that JAK3 could be an exploitable target for protecting against periodontal bone loss by restraining the oral bacteria-induced inflammatory response. Future studies examining the expression of JAK3, Wnt3, and Dvl3 and their relevance to the predisposition to periodontitis will unravel the potential therapeutic significance of these protein kinases.

Discussion

The anti-inflammatory signaling pathways that counterbalance the inflammatory response to bacterial infections are poorly characterized. Here, we demonstrate that activation of JAK3 restrains the *P. gingivalis*-driven inflammatory response *in vitro,* along with murine periodontal destruction *in vivo*. *P. gingivalis*-activation of JAK3 increases the Wnt3a-Dvl3

signaling axis which, subsequently, restrains the magnitude of the inflammatory response. Further, JAK3-mediated control of Wnt3a-Dvl3 activity occurs through phosphorylation of a ubiquitin E3 ligase which subsequently reduces the ubiquitin-mediated degradation of Wnt3a, enhances Dvl3-induced β-catenin expression, and promotes the activation of NF-κB in innate immune cells. *In vivo* relevance of this anti-inflammatory mechanism was confirmed in a *P. gingivalis* infection-induced mouse model of periodontal inflammation and bone loss. Thus, targeting JAK3 or its downstream signaling proteins may present a novel strategy to manipulate the direction and intensity of inflammation in the context of periodontitis (Fig. 9). Should, propagation of JAK-Wnt3a-Dvl3 signaling prove an important anti-inflammatory strategy in other disease contexts, therapeutic amplification or suppression of the JAK-Wnt3a-Dvl3 axis may prove efficacious in multiple conditions, depending on the clinical necessity.

We previously found that JAK2 plays a role in the production of ROS in response to *P. gingivalis* infection by controlling the activity of NF-κB (54). We have also reported that JAK3 plays a distinct role in maintaining immune homeostasis in TLR4-mediated inflammation (11). Our current findings isolate the anti-inflammatory role of JAK3 from that of other JAK isoforms and confirm its inhibitory role in *in vitro* and *in vivo*. A number of previous studies suggest that inhibition of JAK3 could be a double-edged sword for the progression of immune responses, and play distinct inflammatory roles in innate and adaptive immune cells. JAK3 inhibitors act as an immunosuppressant in the treatment of rheumatoid arthritis, transplant rejection, psoriasis, and other immune-mediated disorders (55-57), but they also aggravate the magnitude of inflammation in innate immunity, and periodontal and intestinal inflammation (10, 15). Kim et al (58) reported that JAK3-engaged IL-4 signaling is required for production of IL-10 leading to down-regulation of LPS-induced ICE activity, and subsequently restraint of IL-1β production, indicating that autocrine IL-10 signaling is an alternative way that JAK3 can be employed to restrain inflammation. Our previous study found JAK3 also affected activity of the LPS-induced PI3K-Akt pathway through which IL-10 production was controlled (11). Combined with the current findings that Wnt3a-Dvl3 is a target of JAK3, we have established that JAK3 could function as a pivotal hub in host anti-inflammatory responses, and dynamically regulate inflammation through a hierarchical and intertwined signaling network. On the other hand, JAK3 is not the only molecule with opposing effects in innate and adaptive immunity. Other signaling proteins, such as mTORC1 and Akt1, have been widely reported to have opposing effects in innate and adaptive immunity through undetermined mechanisms (59-61). Considering the essential role of these kinases in cell proliferation, their distinct function in innate and adaptive immunity might have evolved to meet the needs of the host immune response in different cell types. Taken together, our findings in this study illustrate the complicated network the host employs to orchestrate immune responses to bacterial challenge. Further investigations into the regulatory activity of JAK3 in different spatial and temporal contexts is likely to yield greater insight into the host response in general.

In this study, our results have shown a discrepancy in TNFα and IL-6 production between *in vitro* and *in vivo* assays. JAK3 inhibition significantly enhanced the production of IL-6, IL-12, and TNFα in *P. gingivalis*-simulated cultured cells, while only IL-6 was significantly increased in the gingival tissue from *P. gingivalis*-infected mice. This discrepancy might be

due to the phase of inflammation, differential dynamics of cytokines synthesis, variant resistance to *P. gingivalis*-induced tolerance, mutual regulation of cytokines, and importantly, the different components of samples. In our animal model, the gingival tissues for TNFα and IL-12 analysis were from the mice post-infection with *P. gingivalis* for 42 days, which is approximately equal to 6 years' infection in humans. Compared to the cultured cells challenged with *P. gingivalis* overnight, it is not surprising to see the different phase of inflammation leading to the different levels of TNFα and IL-12. Moreover, TNFα and IL-12 are produced more quickly and last a shorter time as compared with IL-6 in many inflammation models (62, 63), which could be another reason for being unable to observe JAK3 inhibition of TNFα and IL-12 from gingival tissue. In addition, previous studies have shown that TNFα and IL-12 are more sensitive than IL-6 to the chronic activation of TLR-mediated immune cell tolerance (64, 65). Since the mice in our model were infected with *P. gingivalis* for 12 days, it is also reasonable to speculate that immune cell tolerance in gingival tissue could lead to the non-difference of TNFα and IL-12 in gingival tissue. Furthermore, increased IL-6 has been reported to suppress TNFα production in *R. aurantiacus*-induced granulomatous and inflammatory response in the early phase of infection (66), which could also have occurred in the *P. gingivalis* infected mice. Lastly, the difference of the samples we used *in vitro* and *in vivo* could also be a major reason for this discrepancy. Unlike an *in vitro* monoculture, gingival tissue includes not only monocytes and macrophages, but also neutrophils, adaptive immune cells, epithelial cells, fibroblasts and other cells. Although we didn't examine the function of JAK3 in adaptive immune cells from gingival tissue, the opposite regulatory function of JAK3 in innate and adaptive immune cells has been extensively reported (8, 10, 11) and thus possibly affects the final readout of the JAK3 net effect on TNFα and IL-12 production. Of note, as IL-6 is a strong inducer of RNAKL and crucial to the differentiation of Th17, as well as the polarization of macrophages(67-69), additional players might be involved in *P. gingivalis*-induced bone loss. Due to the limited size of murine gingival tissue, it is difficult for us to isolate monocytes or macrophages, as well as adaptive immune cells to test the possible distinct function of JAK3 in single types of immune cells. Future research focusing on the production of inflammatory cytokines at different phases of *P. gingivalis* infection, and using single cell sequencing analysis from pooled tissues or cervical lymph nodes if possible, will likely provide more interesting information.

P. gingivalis infection has been reported to activate Smad ubiquitin regulatory factor 1 (Smurf1) and thus promote the proteasomal degradation of MyD88, leading to a reduction in bacterial clearance (29). Moreover, the Smurf 1-mediated degradation of MyD88 is specific to *P. gingivalis* infection. Other E3 ligases involved in the regulation of TLR signaling pathways, such as Triad3A and Pellino-1, do not mediate any change in MyD88 (29). A recent study showed that the ubiquitin-modifying enzyme A20 (also known as TNFα-inducible protein 3) is also involved in *P. gingivalis*-induced inflammation (70, 71), indicating a potentially general influence of *P. gingivalis* on the activity of ubiquitin E3 ligases. In this study, we demonstrate that *P. gingivalis* infection induces the phosphorylation of Nedd4-2 leading to its inactivation in innate immune cells. Considering that both Nedd4-2 and Smurf1 are members of the HECT family, these results are consistent with an extensive modulatory function of *P. gingivalis* on ubiquitin E3 ligases and suggest that the HECT

family could be a target of *P. gingivalis*. Notably, *P. gingivalis* activates Smurf1 through a *de novo* synthesized cytokine, TGF-β1, which is produced in a C5aR/TLR2-dependent manner, and we found that *P. gingivalis* infection leads to the phosphorylation of Nedd4-2 in the very early stage of infection. These results suggest that *P. gingivalis* could manipulate the ubiquitination system in distinct and comprehensive ways. From an evolutionary perspective, *P. gingivalis* could dynamically exploit host cell machinery to benefit its survival by upregulating anti-inflammatory processes, such as Nedd4-2-mediated Wnt3 signaling, at the early stage of infection, and then reducing bacterial clearance molecules, such as MyD88, at later stages.

Wnt3a is a protein that is mainly produced by epithelial cells and functions through either paracrine or autocrine pathways. After binding to the Wnt3a receptor (LRP) and completing its function, most Wnt3 protein is phagocytosed and subjected to ubiquitin-mediated degradation. While we found that *P. gingivalis* infection enhances the ubiquitination of Wnt3a through JAK3-mediated Nedd4-2 phosphorylation, how Wnt3a protein is degraded and where this degradation event occurs, for example, in the ER, during transportation by the Golgi, or by targeting of the free form of Wnt3a in the cytosol, remains unknown. Thus, our results indicate that *P. gingivalis* infection regulates Wnt3a degradation by targeting the general/whole Wnt3 protein cycle, at least in the innate cells we tested. Considering the critical function of Wnt signaling in the regulation of the degradation of other proteins (72), further research to elucidate the dynamics of the ubiquitin-mediated degradation of Wnt3a in *P. gingivalis*-infected cells would provide novel insights into Wnt protein trafficking.

P. gingivalis modification of the phosphorylation and ubiquitination host cell machinery is well established; however, the interaction between phosphorylation and ubiquitination in *P. gingivalis*-infected cells has not been investigated. In this study, we found that the *P. gingivalis*-induced phosphorylation of JAK3 and Nedd4-2 alters the ubiquitination of Wnt3a and thus affects the inflammatory responses. However, our results cannot exclude the involvement of other inflammatory signaling pathways activated by *P. gingivalis*-induced JAK3 phosphorylation. Since the phosphorylation of protein kinases has been shown to modify ubiquitination activity in other contexts (73, 74), it is possible that *P. gingivalis*-induced phosphorylation and ubiquitination employ a “multiple to multiple” rather than an “individual to individual” pattern. Thus, our phosphorylation and ubiquitination interaction results highlight the importance of global omics research into *P. gingivalis*-induced phosphorylation of kinases and ubiquitin ligases.

In summary, we have established that *P. gingivalis*-induced activation of Wnt3a signaling through JAK3 is an important anti-inflammatory mechanism in the maintenance of innate immune homeostasis. Moreover, we show that the JAK3-mediated inhibition of Nedd4-2 controls the expression of Wnt3a and its downstream molecular signaling pathway proteins through ubiquitination-induced proteasomal degradation. Our *in vivo* data using a *P. gingivalis*-induced alveolar bone loss model also confirmed the anti-inflammatory role of JAK3-Nedd4-2-Wnt3a signaling, suggesting that targeting JAK3-mediated Wnt3a signaling, or the expression of ubiquitin E3 ligases, could be a novel strategy to manipulate the intensity and direction of inflammation and thus benefit patients with inflammatory diseases extending beyond periodontitis.

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Abbreviations list

JAK3
Janus kinase 3

Wnt3
Wingless-INT (Wnt) 3

Dsh/Dvl3
Dishevelled-3

Nedd4
Neural precursor cell expressed developmentally down-regulated 4

TLRs
Toll-like receptors

NF-κB
Nuclear factor kappa-light-chain-enhancer of activated B cells

TNFα
Tumor necrosis factor alpha

TGFβ
Transforming growth factor beta

IL (-6, -12, -10, -4, -1Ra)
Interluekin (-6, -12, -10, -4, -1receptor antagonist)

PI3K
Phosphoinositide 3-kinase

Akt
Protein kinase B

mTOR
Mammalian target of rapamycin

MAPK
Mitogen-activated protein kinase

GSK3β
Glycogen synthase kinase 3 beta

STAT
Signal transducer and activator of transcription

PMN
Polymorphonuclear

CEJ-ABC
Cementoenamel junction (CEJ)- alveolar bone crest (ABC)

MyD88
Myeloid Differentiation primary response 88

HECT
Homologous to the E6-AP Carboxyl Terminus

IP
Immunoprecipitation

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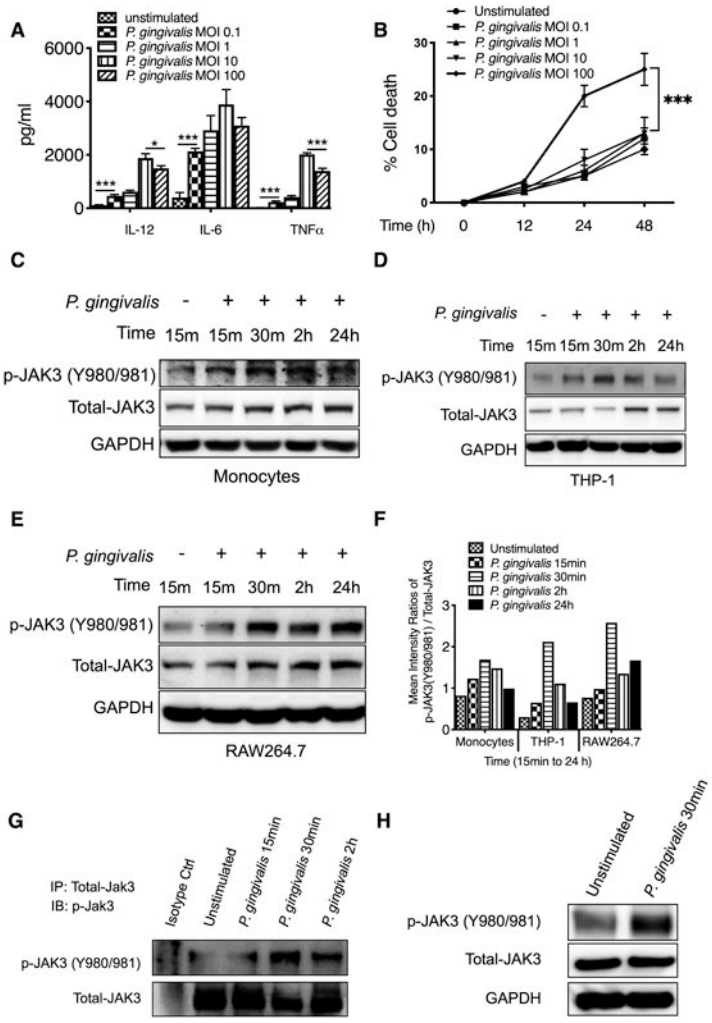

Figure 1. *P. gingivalis* challenge robustly enhances the activity of JAK3 in *P. gingivalis*-stimulated innate immune cells

Human monocytes were stimulated with *P. gingivalis* at different MOIs (1, 10, 50, and 100) for 24 h, and a trypan blue exclusion assay was performed at various time intervals. (A) The production of proinflammatory cytokines (TNFα, IL-6, and IL-12P40) was determined by ELISA after 24 h of stimulation. (B) Percentage of dead cells after challenge of *P. gingivalis* with different MOIs. (C to F) Whole-cell lysates of human monocytes (C), THP-1 (D), and RAW264.7 cells (E) challenged with *P. gingivalis* (MOI of 10) analyzed by Western blot. Immunoblots were probed with antibodies to phospho- and total JAK3 and GAPDH. (F) The mean intensity ratios of phospho- to total- JAK3 were determined by densitometry. (G, H) JAK3 was immunoprecipitated from the cell lysates of *P. gingivalis*-stimulated human monocytes. Cross-linking and freeze-drying were also used to purify and enrich proteins in immunoprecipitates. Western blotting was used to determine the phosphorylation of JAK3 in regular (G) and enriched (H) immunoprecipitates. All the blots shown are representative of three to five biological replicates. For A and B, the data represent the arithmetic mean ±standard deviation (S.D.) of three independent experiments. *, and *** indicate statistical significance at $P<0.05$ and $P<0.001$, respectively.

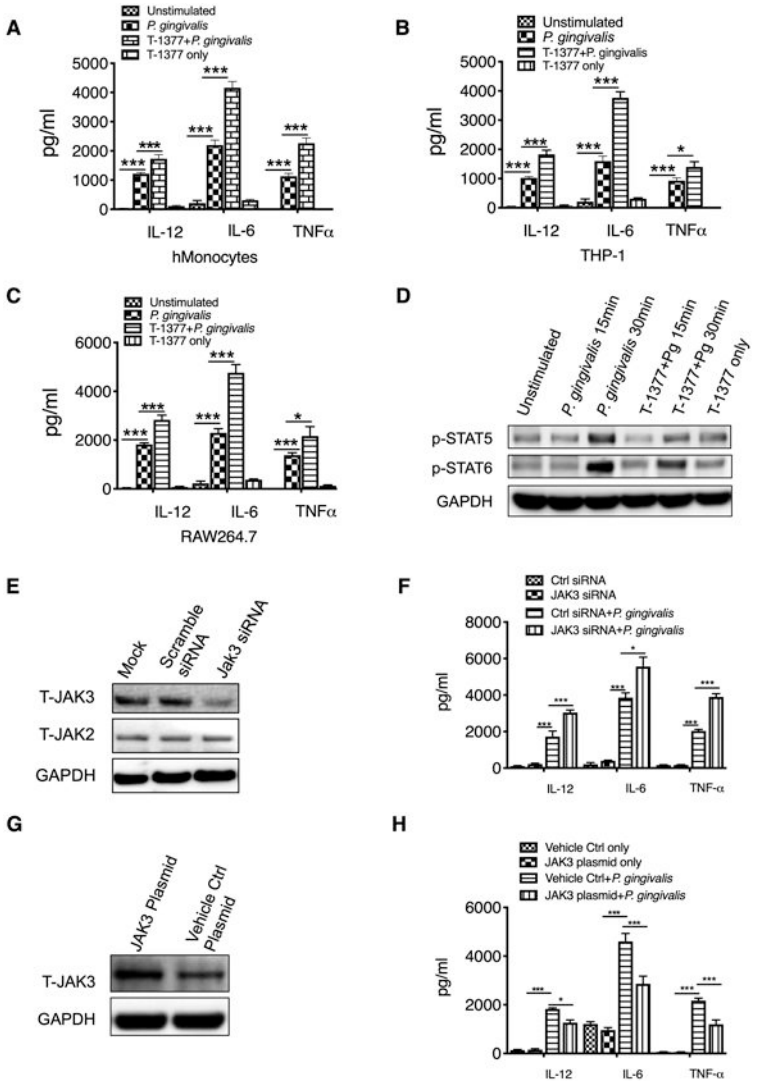

Figure 2. JAK3 negatively regulates *P. gingivalis*-induced proinflammatory cytokine production in innate immune cells

Cells were pretreated with a JAK3 inhibitor (T-1377, 1 nM) for 2 h and then stimulated with *P. gingivalis* over a 24 h time course. (A to C) Levels of TNFα, IL-6, and IL-12P40 in cell-free supernatants produced by human monocytes (A), THP-1 (B) and RAW 264.7 cells (C). (D) Total cell lysates were collected at the indicated times and probed for phosphorylated STAT5 and STAT6. (E to H) Human monocytes were transfected with specific siRNA or a plasmid to silence or overexpress JAK3, respectively. Nontargeting siRNA and an empty vector were used as controls. After transfection for 72 h, human monocytes were stimulated with *P. gingivalis* over a 24 h time course. The cell-free supernatant and whole-cell lysates were collected to determine the levels of inflammatory cytokines and the transfection efficiency, respectively. The transfection efficiency was estimated by the expression levels of total JAK3. JAK3 silencing (E) significantly enhances the production of TNFα, IL-6, and IL-12 (F), while the overexpression of JAK3 (G) significantly decreases the levels of TNFα, IL-6, and IL-12 in *P. gingivalis*-stimulated human monocytes (H). For D, F, and G, the blots shown are representative of three to five biological replicates. Other data represent the

arithmetic mean ± S.D. of three independent experiments. *, and *** indicate statistical significance at $P<0.05$ and $P<0.001$, respectively.

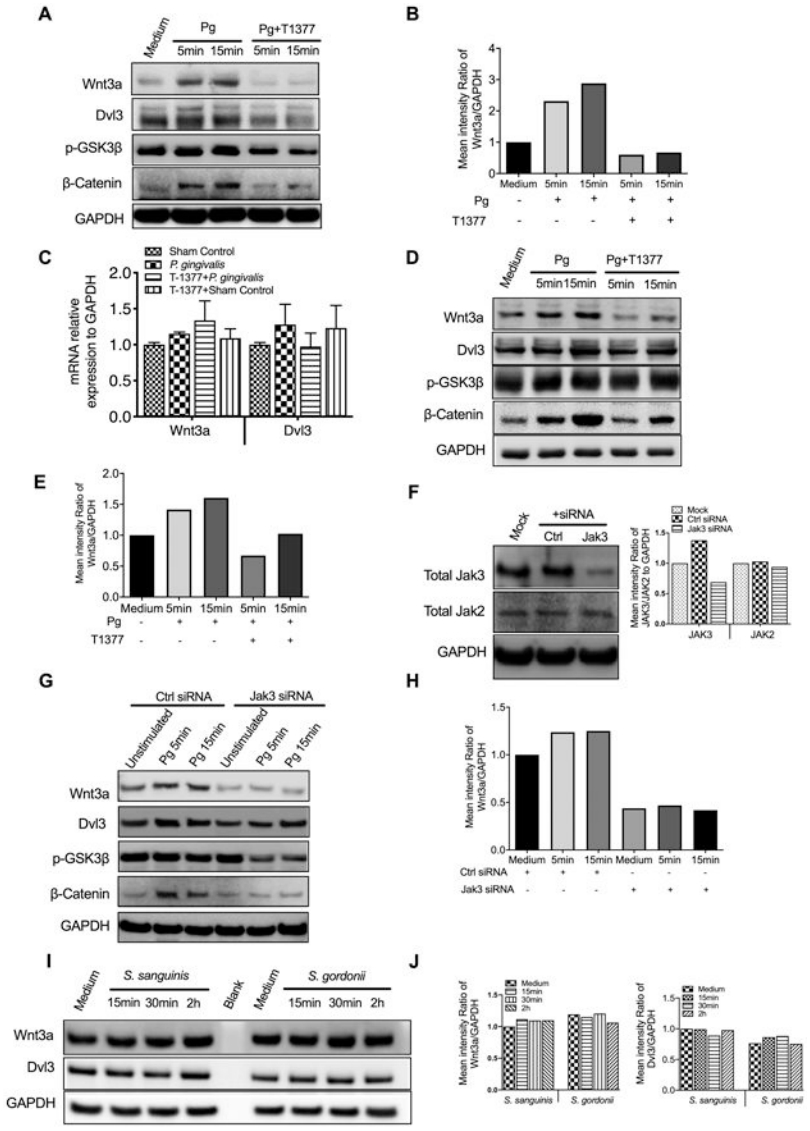

Figure 3. *P. gingivalis* enhances the activity of Wnt3a-β-catenin signaling through JAK3 in innate immune cells

Purified human monocytes (A to C) and THP-1 cells (D, E) were pretreated with a JAK3 inhibitor (1 nM T-1377) for 2 h and then stimulated with *P. gingivalis* (MOI of 10) for the indicated time. (A to E) Total cell lysates were probed for Wnt3a, Dvl3, p-GSK3β, p-β-catenin, and GAPDH (A, D) and mRNA levels of Wnt3a and Dvl3 were also detected after challenge with *P. gingivalis* for 2 h using qRT-PCR (C). (F to H) Western blot of human monocytes transfected with nontargeting or JAK3-specific siRNA for 72 h and then stimulated with *P. gingivalis* for the indicated time. Blots were probed with antibodies against Wnt3a, Dvl3, p-GSK3β, p-β-catenin, and GAPDH (G). The intensity ratio of each protein relative to that of GAPDH was determined by densitometry (B, E, F, H). (I, J) THP-1 cells were stimulated with *Streptococcus sanguinis* and *Streptococcus gordonii* (MOI of 10) for the indicated time. Total cell lysates were probed for p-JAK3, Wnt3a, Dvl3, and GAPDH. All the blots shown are representative of three to five biological replicates.

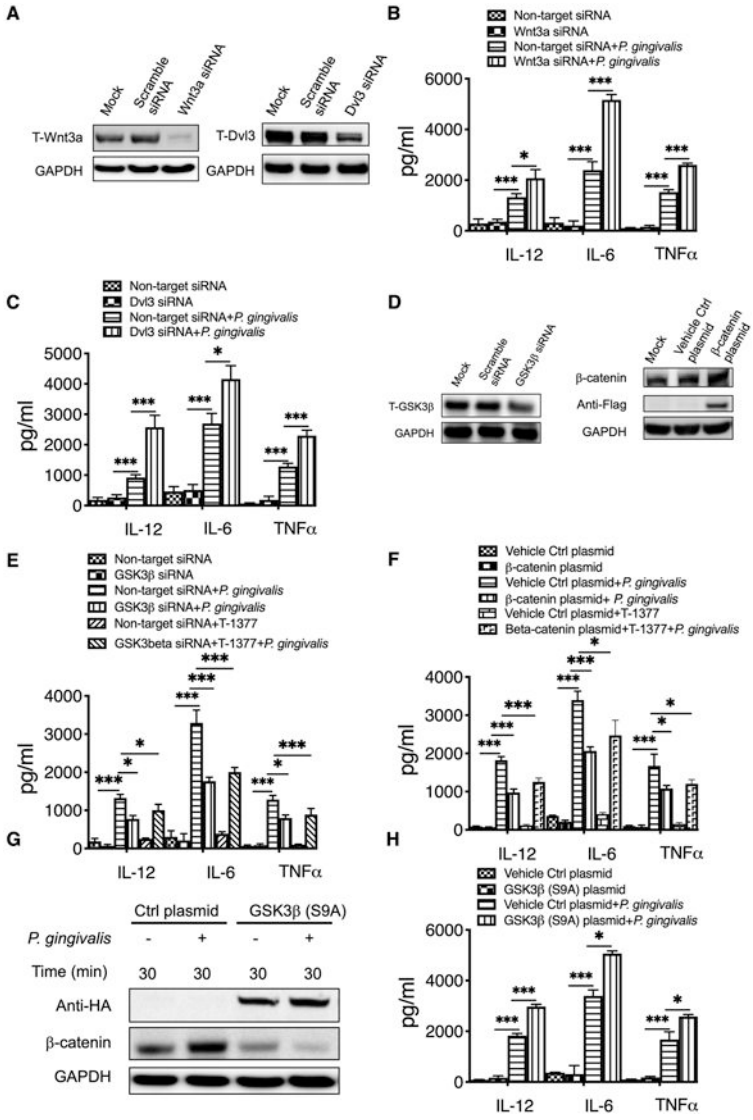

Figure 4. JAK3-mediated Wnt3a-β-catenin signaling suppresses the production of inflammatory cytokines in *P. gingivalis*-stimulated innate immune cells

Purified human monocytes were transfected with nontargeting control, Wnt3a, Dvl3, or GSK3β siRNAs, or a plasmid encoding β-catenin or a serine 9 to alanine constitutively active GSK3β mutant for 72 h and then stimulated with *P. gingivalis* over a 24 h time course in the absence or presence of a JAK3 inhibitor (1 nM T-1377). Cell-free supernatants and total cell lysates were harvested to determine the levels of TNFα, IL-6, and IL-12P40 and the transfection efficiency, respectively. (A to C) The silencing of Wnt3a or Dvl3 (A) significantly enhances the production of TNFα, IL-6, and IL-12P40 (B, C) in *P. gingivalis*-stimulated monocytes. The silencing of GSK3β or overexpression of β-catenin (D) abrogates the influence of JAK3 inhibition on the production of inflammatory cytokines and significantly reduces the levels of TNFα, IL-6, and IL-12P40 in *P. gingivalis*-stimulated monocytes (F). (G, H) Total cell lysates were probed with antibodies against β-catenin and the inserted HA tag to determine the transfection efficiency and its effect on the expression of β-catenin (G). (H) The production of TNFα, IL-6, and IL-12P40 in *P. gingivalis*-

stimulated human monocytes overexpressing the GSK3β mutant. All the blots shown are representative of three to five experiments. For B, C, E, F, H, all the data were generated in the same experiment and represent the arithmetic mean±S.D. of three independent experiments. *, and *** indicate statistical significance at $P<0.05$ and $P<0.001$, respectively.

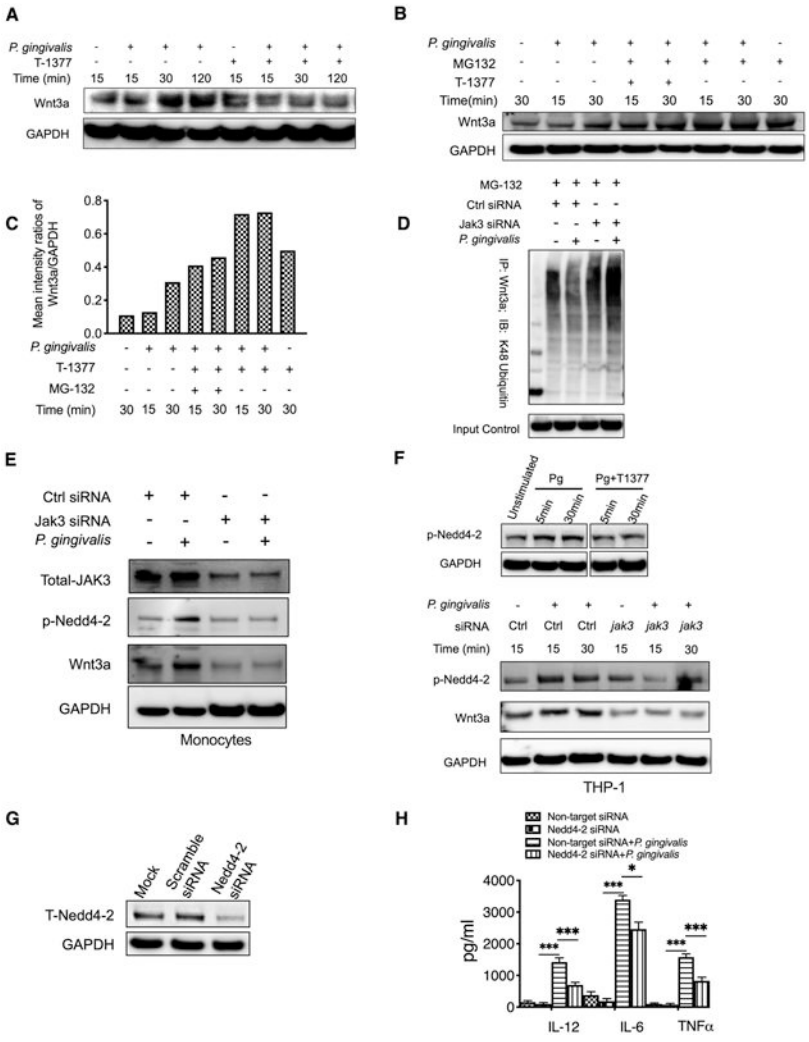

Figure 5. JAK3 controls the expression of Wnt3a through the phospho-inactivation of Nedd4-2 and enhances the ubiquitination of Wnt3a

Purified human monocytes were stimulated with *P. gingivalis* and total cell lysates were probed for the levels of Wnt3a with or without pretreatment of JAK3 inhibitor, T-1377 (1 nM) (A, B), or proteasome inhibitor, MG-132 (10 μM) (B, D). (C) The intensity ratio of Wnt3a to GAPDH was determined by densitometry. JAK3- specific siRNA was used to silence JAK3 in human monocytes (D, E) and THP-1 cells (F), and the total lysate of *P. gingivalis*-stimulated human monocytes was probed for the levels of immunoprecipitated Wnt3a and K48 ubiquitinated Wnt3a (D), the phosphorylation of Nedd4-2, and the levels of total Wnt3a and GAPDH (E, F). (F) JAK3 inhibitor (1nM T-1377) was also used to determine the effect of JAK3 on the phosphorylation of Nedd4-2 in *P. gingivalis*-stimulated THP-1 cells. (G to H) Purified human monocytes were transfected with nontargeting control siRNA or Nedd4-2-specific siRNA for 72 h and then stimulated with *P. gingivalis* over a 24 h time course. (G, H) Silencing of Nedd4-2 (G) significantly reduces the levels of TNFα, IL-6, and IL-12P40 in *P. gingivalis*-stimulated monocytes (H). All the blots shown are representative of three to five biological replicates. The data in (H) represent the arithmetic

mean±S.D. of three independent experiments. *, and *** indicate statistical significance at $P<0.05$ and $P<0.001$, respectively.

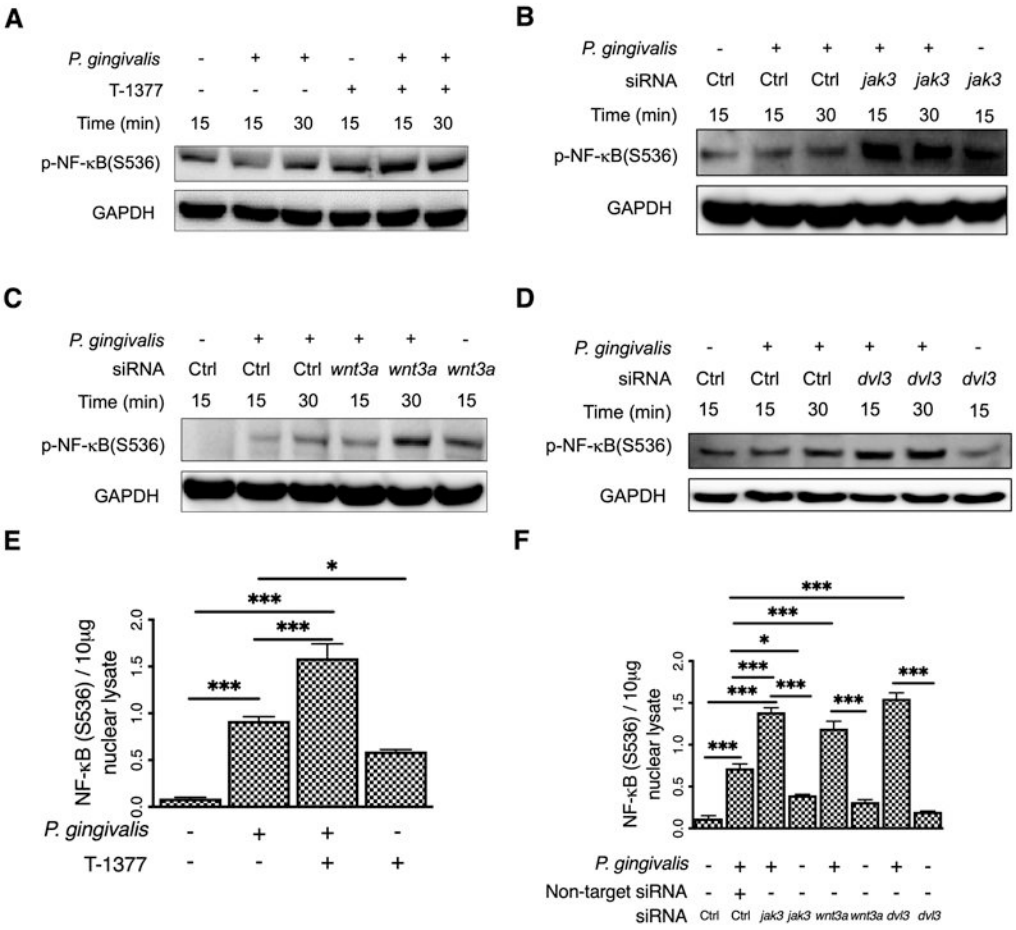

Figure 6. Inhibition of JAK3 or Wnt3 signaling increases *P. gingivalis*-induced NF-κB activity

Purified human monocytes were pretreated with T-1377 for 2 hours or transfected with nontargeting control, or JAK3-, Wnt3a- or Dvl3-specific siRNA for 72 h and then stimulated with *P. gingivalis* for the time indicated. Total cell lysates and nuclear lysates were harvested from separate groups. (A to D) Western blot of total cell lysates probed for the levels of phospho-NF-κB (Ser536) and GAPDH (D). (E, F) Ten micrograms of nuclear lysate were used to determine the transcription factor binding levels of NF-κB in human monocytes stimulated with *P. gingivalis* for 6 h. For A-D, the blots shown are representative of three to five experiments. For E and F, the data represent the arithmetic mean±S.D. of three independent experiments. *, and *** indicate statistical significance at $P<0.05$ and $P<0.001$, respectively.

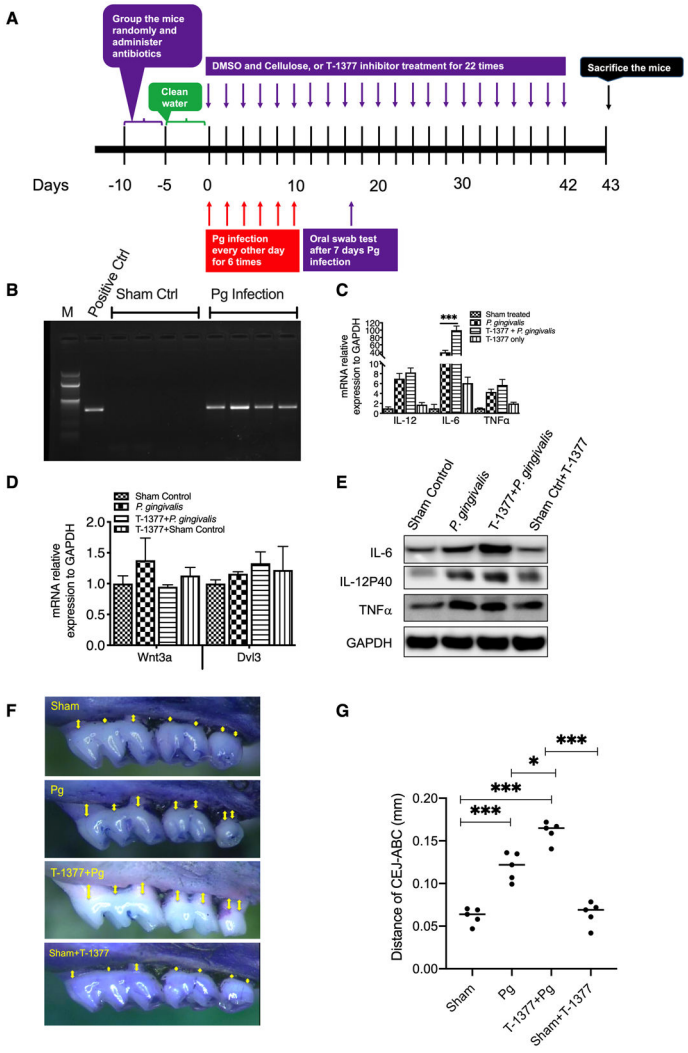

Figure 7. JAK3 restrains inflammation in periodontal tissues and aggravates the severity of bone loss in *P. gingivalis*-infected mice

(A, B) Eight- to 12-week-old C57BL/6 mice were divided randomly into one sham control group and two experimental groups (n=5 per group). The sham control group was treated with cellulose and 0.01% DMSO. The experimental groups were orally infected with *P. gingivalis* with or without pretreatment with the JAK3 inhibitor T-1377 (15 mg/kg) (A). Samples from the mouse oral cavity were examined by quantitative nested PCR to confirm *P. gingivalis* infection. (B) Representative electrophoresis images showing *P. gingivalis* DNA obtained from oral samples. (C, D) mRNA levels of TNFα, IL-6, and IL-12P40 (C), as well as Wnt3a and Dvl3 (D) were determined by RT-qPCR. (E) Total lysates of gingival tissue from upper jaws were probed for TNFα, IL-6, IL-12P40, and GAPDH. (F) Alveolar bone loss visualized by methylene blue/eosin staining and typical maxillae from sham-infected; *P. gingivalis*-infected; and T-1377-treated *P. gingivalis*-infected mice are presented. (G) Quantification of *P. gingivalis*-induced bone loss representing the distance from the CEJ to the ABC. Data are presented as the mean CEJ-ABC distance in mm±S.D.; n=5 mice per group. Error bars represent the S.D. *, and *** indicate statistical significance at $P<0.05$ and

$P<0.001$, respectively. Data represent the arithmetic mean±S.D. of three independent experiments.

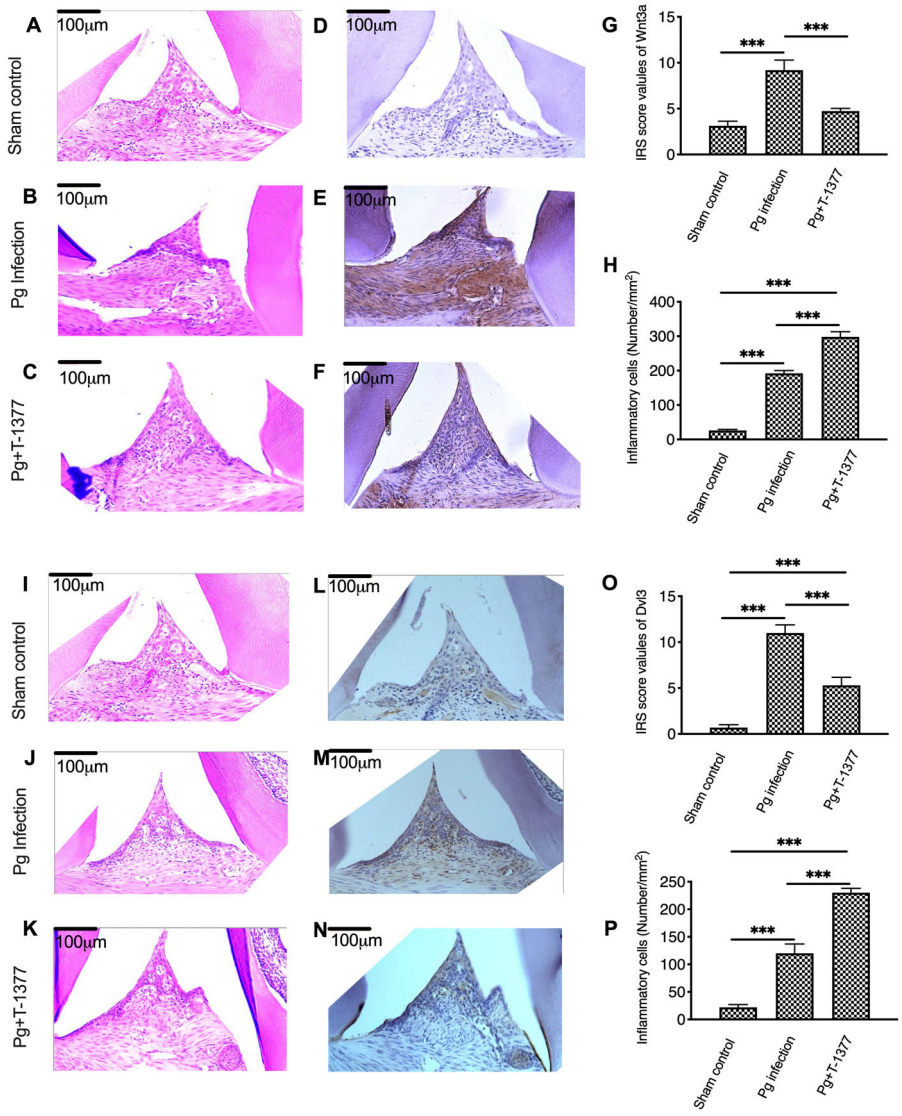

Figure 8. JAK3 inhibition reduces the expression of Wnt3 and Dvl3 in periodontal tissue in *P. gingivalis*-infected mice

Immunohistochemistry staining of serial sections of gingival tissues from experimental mice treated with *P. gingivalis*, or *P. gingivalis* plus JAK3 inhibitor (T-1377, 15mg/kg), or sham control mice treated with DMSO and Cellulose with or without T-1377, showing the expression of Wnt3a (A to F) and Dvl3 (I to N). (G, O, H, P) The mean pixel intensity of Wnt3a (G) and Dvl3 (H), and the inflammatory cells infiltrated in the gingival tissue of *P. gingivalis*-infected mice (H, P) observed from more than 20 microscope fields of view are presented. Error bars represent the S.D. *, and *** indicate statistical significance at $P<0.05$ and $P<0.001$, respectively. Data represent the arithmetic mean±S.D. of three independent experiments.

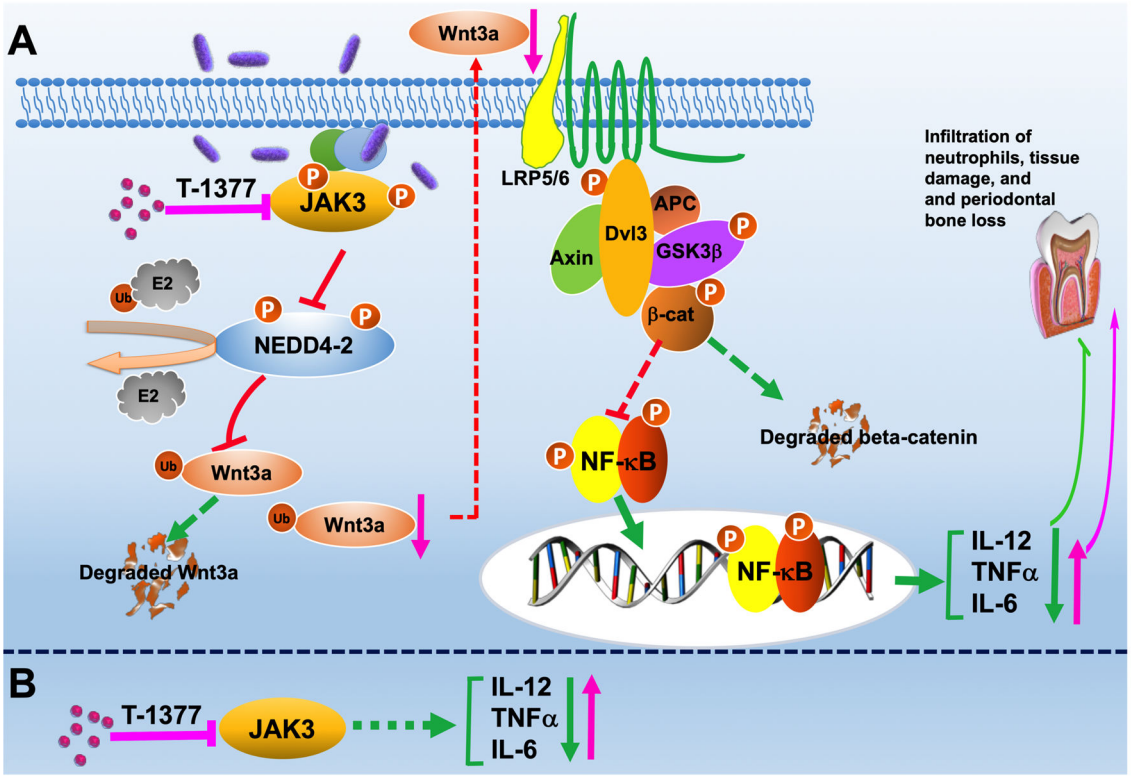

Figure 9. Schematic signaling model of JAK3 restrains *P. gingivalis*-induced inflammation through Wnt3-Dvl3 signaling in innate immune cells

(A) *P. gingivalis* infection activates proinflammatory signaling (not shown here) and thus induces the production of proinflammatory cytokines. Concurrently, JAK3 is phosphorylated upon the challenge of *P. gingivalis* and in turn phospho-inactivates Nedd4-2, leading to the decreased ubiquitination of Wnt3a and a subsequent increase in Wnt3a, Dvl3, and phospho-GSK3β levels, and the accumulation of β-catenin, which diminishes NF-κB signaling and ultimately constrains the proinflammatory immune response. In contrast, JAK3 inhibition promotes the activity of Nedd4-2, decreases the amount of Wnt3a, Dvl3, phospho-GSK3β, and β-catenin, which ultimately increases NF-κB activity, leading to increased proinflammatory cytokine production and inflammatory cell infiltration and the consequent exacerbation of inflammation-induced alveolar bone loss. (B) Briefly, activation of JAK3 is essential to restrain the production of pro-inflammatory cytokines (green) while inhibition of JAK3 leads to a robust increase of these cytokines (pink) in innate immune cells.