Journal of Dental Research
2022, Vol. 101(6) 675–685

DOI: 10.1177/00220345211059418
journals.sagepub.com/home/jdr

Leptin Aggravates Periodontitis by Promoting M1 Polarization via NLRP3

Y. Han[1,2], Y. Huang[1,2], P. Gao[3], Q. Yang[1,2], L. Jia[2,4,5], Y. Zheng[1,2], and W. Li[1,2]

Abstract

Periodontitis is characterized by periodontal pocket formation, loss of attachment, and alveolar bone resorption. Both innate and adaptive immunity are involved in the pathogenesis of this oral chronic inflammatory disease. Accumulating evidence indicates a critical role of leptin in periodontal diseases. However, the mechanism by which leptin promotes periodontitis pathogenesis remains unclear. In the present study, we observed an elevated expression of leptin in the serum of periodontitis mice compared to that in healthy controls. There was a higher extent of M1 phenotype macrophage infiltration in mice periodontitis samples than in healthy controls. A positive correlation was observed between the serum leptin levels and M1 macrophages. Treatment with leptin increased M1 macrophage polarization and decreased M2 macrophage polarization in RAW 264.7 cells. Moreover, leptin facilitated lipopolysaccharide (LPS)–induced M1 phenotype macrophage polarization in RAW 264.7 cells. In bone marrow–derived macrophages (BMDMs) generated from leptin-deficient obese (*ob/ob*) mice, M1 macrophage polarization was significantly attenuated after LPS stimulation compared to the healthy controls. With regards to the molecular mechanism, we found that leptin activated the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome and promoted M1 polarization via the NLRP3 inflammasome in vitro. In BMDMs generated from *Nlrp3*$^{-/-}$ mice, M1 macrophage polarization was significantly attenuated after synchronous stimulation with leptin and LPS compared with BMDMs produced by healthy controls. The NLRP3 inhibitor MCC950 also prevented leptin-mediated M1 macrophage polarization in RAW 264.7 cells. *Nlrp3*$^{-/-}$ periodontitis models indicated that leptin aggravates the periodontal response to the ligature by promoting M1 macrophage polarization via the NLRP3 inflammasome. Taken together, we show that leptin promotes the progression of periodontitis via proinflammatory M1 macrophage skewing, and targeting leptin/NLRP3 signaling may be a feasible approach for treating periodontitis.

Keywords: periodontal diseases, macrophage polarization, NLRP3 inflammasome, alveolar bone loss, inflammation, innate immunity

Introduction

Periodontitis is an inflammatory disease characterized by periodontal pocket formation, loss of attachment, and alveolar bone resorption (Armitage et al. 2003). A strong innate immune response was first stimulated, followed by the acquired immune response (Seymour et al. 1993). The innate immunity is the first line of defense against invading oral pathogens, which mainly consists of the oral epithelial barrier and the activity of phagocytic cells, such as neutrophils, monocytes, and macrophages. These cells migrate to the site of infection in response to chemotaxins and cytokines and then attack and remove invading bacteria to regulate local immune-inflammatory responses (Yang et al. 2018). Naive macrophages (M0) can be polarized into 2 different phenotypes: the classical proinflammatory (M1) class or the anti-inflammatory (M2) class, in response to local physiological or pathological conditions (Sima and Glogauer 2013; Yang et al. 2018). Classically activated M1 macrophages contribute to periodontal tissue destruction (Sima and Glogauer 2013; Gonzalez et al. 2015). In contrast, M2 macrophages promote tissue repair and enhance local cell regeneration (Mantovani et al. 2004; Wu, Chen, et al. 2020).

Leptin, a 16-kDa nonglycosylated protein encoded by the LEP gene (the human homolog of murine Lep, also known as ob), was first discovered in 1994 (Zhang et al. 1994). Leptin is mainly produced by adipocytes and regulates energy metabolism.

[1]Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China
[2]National Center of Stomatology, National Clinical Research Center for Oral Diseases, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing, China
[3]Key Laboratory of Cell Proliferation and Differentiation of the Ministry of Education, School of Life Sciences, Peking University, Beijing, China Peking-Tsinghua Center for Life Sciences, Beijing, China
[4]Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China
[5]Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China

A supplemental appendix to this article is available online.

Corresponding Authors:
W. Li, Department of Orthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun Avenue South, Haidian District, Beijing, 100081, People's Republic of China.
Email: weiranli@bjmu.edu.cn
Y. Zheng, Department of Orthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun Avenue South, Haidian District, Beijing, 100081, People's Republic of China.
Email: yunfei_zheng@bjmu.edu.cn

Previous studies have reported that periodontitis causes increased serum levels of leptin, and its expression is positively correlated with leukocyte and neutrophil counts, as well as levels of serum interleukin (IL)–1β, IL-6, and tumor necrosis factor (TNF) (Gangadhar et al. 2011; Liu et al. 2013; Zimmermann et al. 2013). After periodontal treatment, serum leptin levels decrease significantly (Shimada et al. 2010; Zimmermann et al. 2013). However, the expression of leptin in the gingival crevicular fluid (GCF) is negatively correlated with periodontitis (Karthikeyan and Pradeep 2007; Ahuja et al. 2019). But no systematic study has been conducted to determine the regulatory mechanisms of leptin in periodontitis.

As a circulating hormone, leptin plays a key role in regulating metabolic homeostasis and mediating immune responses by acting on various immune system cells, such as macrophages and natural killer cells. Most studies have indicated that leptin has a proinflammatory effect on macrophages (Conde et al. 2010), while some studies suggest that leptin treatment induces the expression of M2 phenotype surface markers in adipose tissue macrophages (Acedo et al. 2013). Moreover, leptin can indirectly regulate the macrophage phenotype through mast cells (Zhou et al. 2015). Therefore, the effects of leptin on macrophage polarization remain controversial. Whether leptin regulates the development of periodontitis by regulating macrophage polarization and what the underlying mechanisms could be, remain unclear.

In the present study, we investigated the role of leptin in periodontitis development and further clarified the regulatory mechanism of leptin with regards to M1 macrophage polarization. Our results indicate that M1 macrophage polarization, mediated by leptin/NOD-like receptor family pyrin domain containing 3 (NLRP3), may drive inflammatory tissue damage in periodontitis.

Materials and Methods

Cell Culture

A macrophage cell line, RAW 264.7 (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences), was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco), containing 10% fetal bovine serum (FBS; Gibco), 50 U/mL penicillin, and 50 μg/mL streptomycin (Hyclone). All cells were cultured in a humid atmosphere with 95% air, 5% CO_2 at 37°C.

Isolation and Differentiation of Murine Bone Marrow–Derived Macrophages

Tibiae and femora were obtained from wild-type (WT), *Nlrp3*$^{-/-}$, and leptin-deficient obese (*ob/ob*) mice. BMDMs were flushed using RPMI-1640. After red blood cells were lysed, the BMDMs were resuspended in complete RPMI-1640 containing macrophage colony stimulating factor (M-CSF) (30 ng/mL; R&D), 10% FBS, and 1% penicillin for 3 d at 37°C. Then, the cells were harvested and grown in a 6-well plate overnight. To induce polarization, BMDMs were treated with *Escherichia coli*–derived lipopolysaccharide (LPS, 1 μg/mL; Sigma) or leptin (500 μM) for 12 h.

Flow Cytometry

After treatment, the cells were washed and stained for 30 min at 4°C with CD80-FITC (BioLegend) antibodies to characterize the M1 polarization of macrophages. F4/80-APC (BioLegend) was used as a macrophage marker, and $CD80^+/F4/80^+$ cells were considered M1 macrophages. Labeling was quantified using a BD Accuri C6 Flow Cytometer, and data were analyzed with the FlowJo software packages (TreeStar).

Animals

Male, 8-wk-old, *Nlrp3* $^{-/-}$, *ob/ob*, and WT C57BL/6 mice were used for the study. *Nlrp3*$^{-/-}$ and *ob/ob* mice were provided by the School of Life Sciences, Peking University. WT mice were purchased from WeiTong LiHua Co. Animals were maintained under specific pathogen–free conditions. All animal experimental procedures were approved by the Peking University Animal Care and Use Committee (LA2021409) and conformed with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Ligature-Induced Periodontitis Model and Inventions

The ligature-induced periodontitis model was built according to a previous study (Wu, Chen, et al. 2020). Briefly, after general anesthesia, sterile silk ligatures (5–0) were placed around the cervical areas of the upper right second molars of mice. In the control group, the silk ligatures were immediately removed, while in the periodontitis group, the silk ligatures were kept in situ for 7 d.

For the intervention groups, leptin (40 μg/mouse; PeproTech) or vehicle (normal saline) was injected intraperitoneally every day, from 1 d before the ligature-induced periodontitis (Philbrick et al. 2018; Yan et al. 2018). Mice were euthanized by pentobarbital sodium overdose, and the maxilla was carefully removed from the surrounding tissues and fixed in 4% paraformaldehyde for 1 d for further analysis.

Quantitative Reverse Transcriptase Polymerase Chain Reaction and Western Blot

The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and Western blot were conducted as described previously (Han et al. 2021). Details are illustrated in the Appendix. The primers of qRT-PCR used in the experiment are listed in Appendix Table 1.

Histological Analysis of Periodontal Tissues

The details of histological analysis, including hematoxylin and eosin (H&E), immunohistochemical staining, and immunofluorescence staining of periodontal tissues are illustrated in the Appendix.

Enzyme-Linked Immunosorbent Assay Measurement of Serum Leptin

Blood samples were collected by eyeball extraction and centrifuged at 1,200 × *g* for 10 min to obtain serum. Serum leptin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits for animals (MEIMIAN). All kits were used according to the manufacturer's instructions.

Micro–Computed Tomography Analysis

To measure alveolar bone loss, fixed mouse maxillae were scanned using a Skyscan 1174 micro–computed tomography (CT) system (Bruker) at a resolution of 10.8 μm. NRecon and CTvox software were used for the 3-dimensional (3D) reconstruction. The bone volume/tissue volume (BV/TV) ratio was calculated using CTAn software (gray value >1,000). The distance between the cemento-enamel junction and the alveolar bone crest from each side of the upper second molar (mesial, distal, lingual, and buccal sides) was measured on the sagittal and coronal planes, using the Data Viewer software.

Statistical Analysis

SPSS version 19.0 (SPSS, Inc.) was used for the statistical analyses. All data are expressed as the mean ± SD of at least 3 independent experiments. Two-tailed unpaired Student's *t* test was used for 2-group comparisons. One-way analysis of variance (ANOVA) was used for multiple group comparisons. Differences were considered statistically significant at $P < 0.05$.

Results

Increased Serum Leptin Expression and M1 Phenotype Macrophage Infiltration in Periodontitis

We first built ligature-induced periodontitis models to measure serum leptin expression during the pathogenesis of periodontitis. ELISA results showed that serum leptin expression was higher in periodontitis mice than in healthy controls (Fig. 1A). The messenger RNA (mRNA) expressions of IL-6 and TNF were also higher in the diseased mice compared to those in the healthy controls (Appendix Fig. 1A). There were positive correlations between leptin levels and the mRNA expressions of the inflammatory proteins IL-6 and TNF (Fig. 1B). H&E staining showed higher extent of inflammatory cell infiltration in gingival tissues from periodontitis mice, compared with healthy controls (Appendix Fig. 1B). Immunohistochemistry revealed higher expression of IL-1β in periodontitis mice, indicating an inflammatory condition in the periodontium (Appendix Fig. 1B, 1D). Immunofluorescence staining results showed that there was more M1 phenotype macrophage infiltration ($CD86^+/CD68^+$) in the periodontitis mice than in the healthy controls (Appendix Fig. 1C). The number of $CD86^+$ cells in the periodontitis group was higher than that in the control group (Appendix Fig. 1E). There was a positive correlation between leptin levels and the numbers of $CD86^+$ macrophages (per view) (Appendix Fig. 1F).

Leptin Aggravates Mice Periodontitis and Enhances M1 Macrophage Polarization

Next, leptin was intraperitoneally injected into the ligature-induced periodontitis mouse model to investigate the role of leptin in the progression of periodontitis. Micro-CT reconstruction showed that leptin aggravated ligature-induced periodontitis (Fig. 1D). The ligature-induced periodontitis group synchronously treated with leptin showed more severe bone loss with less BV/TV ratio than the untreated ligature-induced periodontitis group (Fig. 1C). H&E staining showed that the distances between the cementum-enamel junction (CEJ) and alveolar bone crest were lower in the ligature combined with leptin treatment group than in the ligature-only group (Fig. 1D). Immunohistochemical staining results consistently showed that IL-1β expression was significantly higher in the leptin treatment group than in the control group (Fig. 1D). Also, we calculated the average optical density (integrated optical density [IOD]/area), which reflects the optical density of per unit area. Consistently, the results showed that there was higher IL-1β expression in the leptin treatment group compared with the control group (Fig. 1F). Moreover, immunofluorescence double staining revealed higher numbers of $CD86^+/CD68^+$ cells in the ligature combined with leptin treatment group than in the ligature group, while the control group exhibited the lowest number of $CD86^+/CD68^+$ cells (Fig. 1E, G).

Leptin Induces M1 Macrophage Polarization in RAW 264.7 Cells

RAW 264.7 cells were treated with leptin for 12 h according to previous studies (Singh et al. 2010; Cao et al. 2016; Hsieh et al. 2017). qRT-PCR results showed that leptin stimulation markedly increased the expression of M1 phenotype macrophage-associated genes, including those of TNF, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) (Fig. 2A), and significantly reduced the expression of M2 phenotype macrophage-associated genes, including those of arginase 1 (Arg-1), dectin, and IL-4 receptor (IL-4R) (Fig. 2B). After stimulation with leptin, the levels of M1 phenotype macrophage-associated protein iNOS increased in a concentration-dependent manner, while the expression of M2 phenotype macrophage-associated protein Arg-1 decreased (Fig. 2C). Next, we chose 500 μM leptin in further experiments. Flow cytometry revealed an elevated percentage of M1 phenotype macrophages (Fig. 2D).

There was more M1 phenotype macrophage infiltration in periodontitis samples, and LPS, the virulence factor to induce inflammatory conditions, activated M1 phenotype macrophage polarization. Above results showed that leptin induced M1 macrophage polarization in RAW 264.7 cells. Therefore, we next explored whether leptin further promoted M1 macrophage polarization under LPS-induced inflammatory microenvironment. qRT-PCR results showed that LPS promoted M1

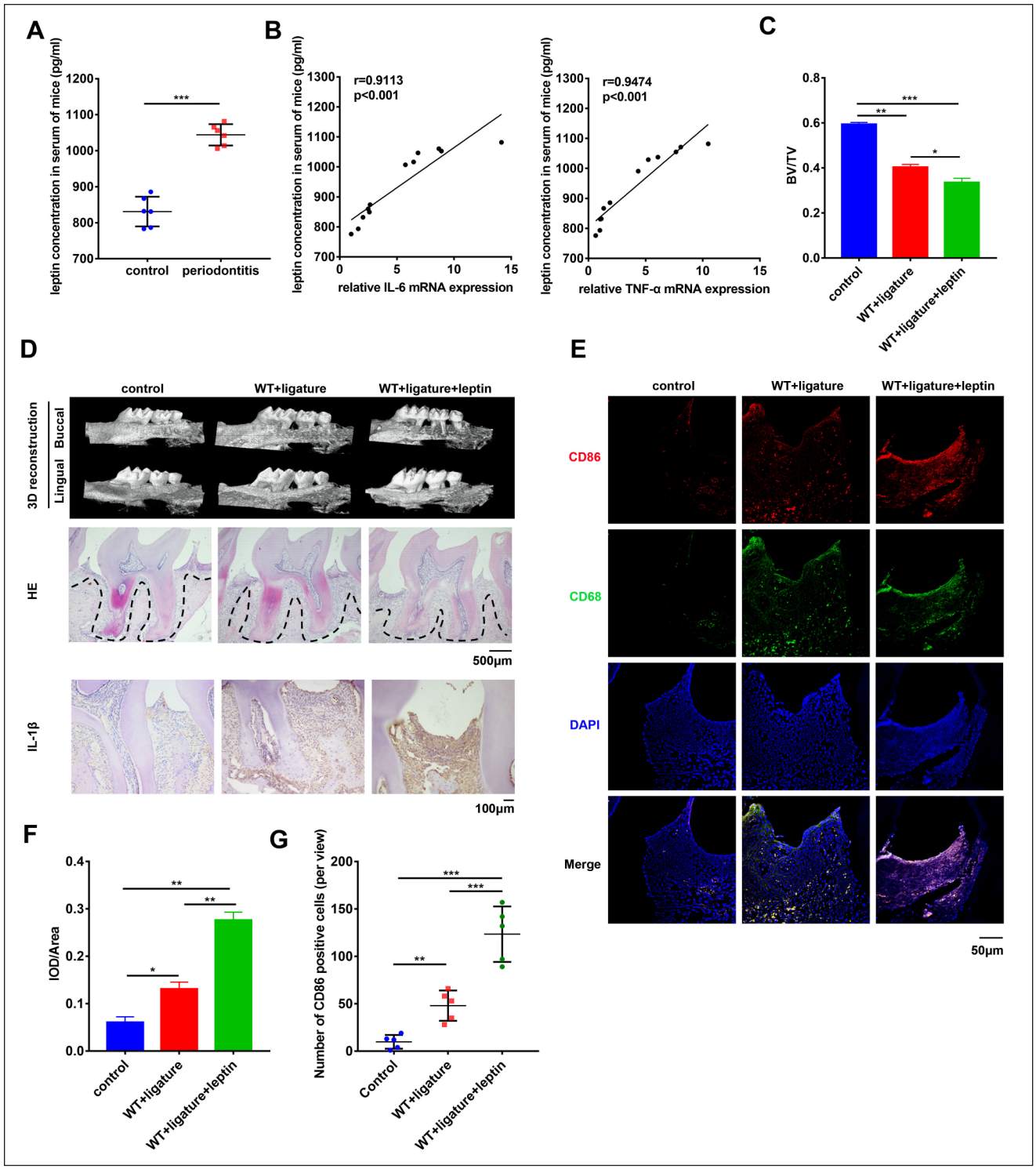

Figure 1. Leptin aggravates mice periodontitis and M1 macrophage polarization. (**A**) Serum leptin expression in mice with periodontitis ($n = 6$) or healthy controls ($n = 6$). The solid lines indicate the median value. (**B**) Correlation analyses between serum leptin concentrations and interleukin (IL)–6 and tumor necrosis factor (TNF) messenger RNA (mRNA) expressions of periodontium of mice in both periodontal and control groups. (**C**) Bone volume/tissue volume (BV/TV) analysis of control, wild-type (WT) + ligature, and WT + ligature + leptin groups ($n = 5$/group). (**D**) Reconstructed 3-dimensional (3D) micro–computed tomography (CT) images, hematoxylin and eosin staining (scale bar = 500 μm), and immunohistochemistry staining (scale bar = 100 μm) of IL-1β of control, WT + ligature, and WT + ligature + leptin groups ($n = 5$/group). (**E**) Immunofluorescence staining (scale bar = 50 μm) showing colocalization of CD86 (M1) positive cells with infiltrated macrophage ($CD68^+$) in the gingival tissues of control, WT + ligature, and WT + ligature + leptin groups ($n = 5$/group). (**F**) Quantitative analysis (integrated optical density [IOD]/area) of IL-1β immunohistochemistry staining of control, WT + ligature group, and WT + ligature + leptin groups. (**G**) The number of M1 phenotype macrophage ($CD86^+/CD68^+$) of the 400× magnification field of control, WT + ligature, and WT + ligature + leptin groups. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

macrophage polarization, which was further enhanced by the synergy between leptin and LPS (Appendix Fig. 2A). At the protein level, the combined treatment with leptin and LPS synergistically increased the expression of M1 marker (Appendix Fig. 2B). Flow cytometry results showed that the expression of CD80 increased in response to combined leptin and LPS treatment, compared to the treatment with LPS alone (Appendix Fig. 2C, D). Immunofluorescence results of CD86 further confirmed the synergistic effect of combined leptin and LPS treatment (Appendix Fig. 2E).

Knockout of Leptin Inhibits LPS-Induced M1 Macrophage Polarization

BMDMs derived from *ob/ob* mice were used to further explore the effect of leptin on LPS-induced M1 macrophage polarization. Knockout of leptin slightly increased the mRNA expression of inflammatory cytokines compared to WT BMDMs. After LPS stimulation, the mRNA expression of M1 phenotype macrophage-associated genes in BMDMs derived from *ob/ob* mice was less compared to WT BMDMs (Fig. 3A). The expression of the M1 macrophage-associated protein iNOS was also significantly reduced (Fig. 3B). Flow cytometry and immunofluorescence staining revealed decreased percentages of M1 phenotype macrophages in BMDMs generated from *ob/ob* mice compared to WT BMDMs after LPS stimulation (Fig. 3C, D).

Leptin Promotes M1 Polarization via NLRP3 Inflammasome

Previous studies have reported that the NLRP3 inflammasome mediates M1 macrophage polarization (Zhang et al. 2020), and leptin can induce NLRP3 inflammasome activation (Lin et al. 2020; Yu et al. 2020). We hypothesized that leptin might promote M1 macrophage polarization by activating the NLRP3 inflammasome. Western blot results showed that leptin activated the NLRP3 inflammasome through the release of activated IL-1β (Appendix Fig. 3A). It also increased the protein expression of NLRP3. However, in BMDMs generated from *ob/ob* mice, this effect was significantly attenuated compared with that in WT BMDMs after LPS and adenosine triphosphate (ATP) stimulation (Appendix Fig. 3B).

Next, BMDMs derived from *Nlpr3*$^{-/-}$ mice were used to explore whether leptin promotes M1 polarization in an NLRP3 inflammasome-mediated manner. There were no physiological changes between WT and *Nlrp3*$^{-/-}$ mice in mRNA levels of inflammatory cytokines (Appendix Fig. 4). Knockout of *Nlrp3* markedly decreased the expression of M1 phenotype macrophage-associated genes in BMDMs derived from *Nlrp3*$^{-/-}$ mice compared to WT BMDMs after synchronous stimulation with leptin and LPS (Fig. 4A). The expression of the M1 macrophage-associated protein iNOS was also markedly reduced (Fig. 4B). Flow cytometry and immunofluorescence staining results revealed a decreased percentage of M1 phenotype macrophages in BMDMs generated from *Nlrp3*$^{-/-}$ mice compared with WT BMDMs, after combined treatment with leptin and LPS (Fig. 4C, D).

Moreover, the NLRP3 inflammasome activation inhibitor MCC950 (1 μM; MedchemExpress) was used to treat RAW 264.7 cells, to further determine whether the M1 macrophage differentiation observed in response to leptin was dependent on NLRP3 inflammasome activation. qRT-PCR, Western blotting, flow cytometry, and immunofluorescence results showed an inhibitory effect of MCC950 for M1 differentiation induced by combined treatment of LPS and leptin (Appendix Fig. 5). These data suggest that NLRP3 inhibition prevents leptin-mediated M1 polarization.

Leptin Aggravates Periodontitis Progression via NLRP3 Inflammasome-Mediated M1 Polarization

WT and *Nlrp3*$^{-/-}$ mice were used to explore the role of NLRP3 in leptin-induced ligature-induced periodontitis. Consistent with the above results, with leptin intraperitoneal injection, *Nlpr3*$^{-/-}$ mice showed less bone loss compared to the WT periodontitis mice (Fig. 5A, B). H&E staining results demonstrated that the perpendicular distance from the alveolar bone crest to the CEJ was less in *Nlpr3*$^{-/-}$ mice than in the WT periodontitis mice, both on the mesial and distal sides (Fig. 5A, C). Immunohistochemical staining results showed that IL-1β expression was significantly lower in *Nlpr3*$^{-/-}$ mice than in the WT periodontitis mice (Fig. 5A). Moreover, immunofluorescence double staining revealed significantly lower amounts of $CD86^{+}/CD68^{+}$ cells in *Nlrp3*$^{-/-}$ mice than in the WT periodontitis mice (Fig. 5A). These results validate the model in which leptin aggravates periodontitis progression via NLRP3 inflammasome-mediated M1 polarization.

Discussion

Recent studies have shown that increased leptin expression can be a diagnostic biomarker for various inflammatory and immune diseases (Abella et al. 2017; La Cava 2017). In addition, previous studies showed that serum leptin levels were positively associated with periodontitis, while GCF leptin levels were negatively correlated with periodontitis (Ahuja et al. 2019), indicating leptin may be involved in the pathogenesis of periodontitis. However, the precise role and regulatory mechanisms of leptin in periodontitis progression remain unclear. Our study showed that intraperitoneal injection of leptin in mice aggravated ligature-induced periodontitis.

Periodontitis is a multifactorial disease caused by the host immune and inflammatory response, which leads to the destruction of periodontal tissues, accompanied by extensive macrophage infiltration (Huang et al. 2016). Macrophages can be polarized into different phenotypes in response to multiple stimulations (Su et al. 2015). The cell walls of gram-negative bacteria contain LPS, which leads to differentiation of macrophages into the M1 phenotype (Talari et al. 2015; Hou et al. 2017). Extensive infiltration of M1 macrophages leads to severe bone destruction in the periodontium (Sima and

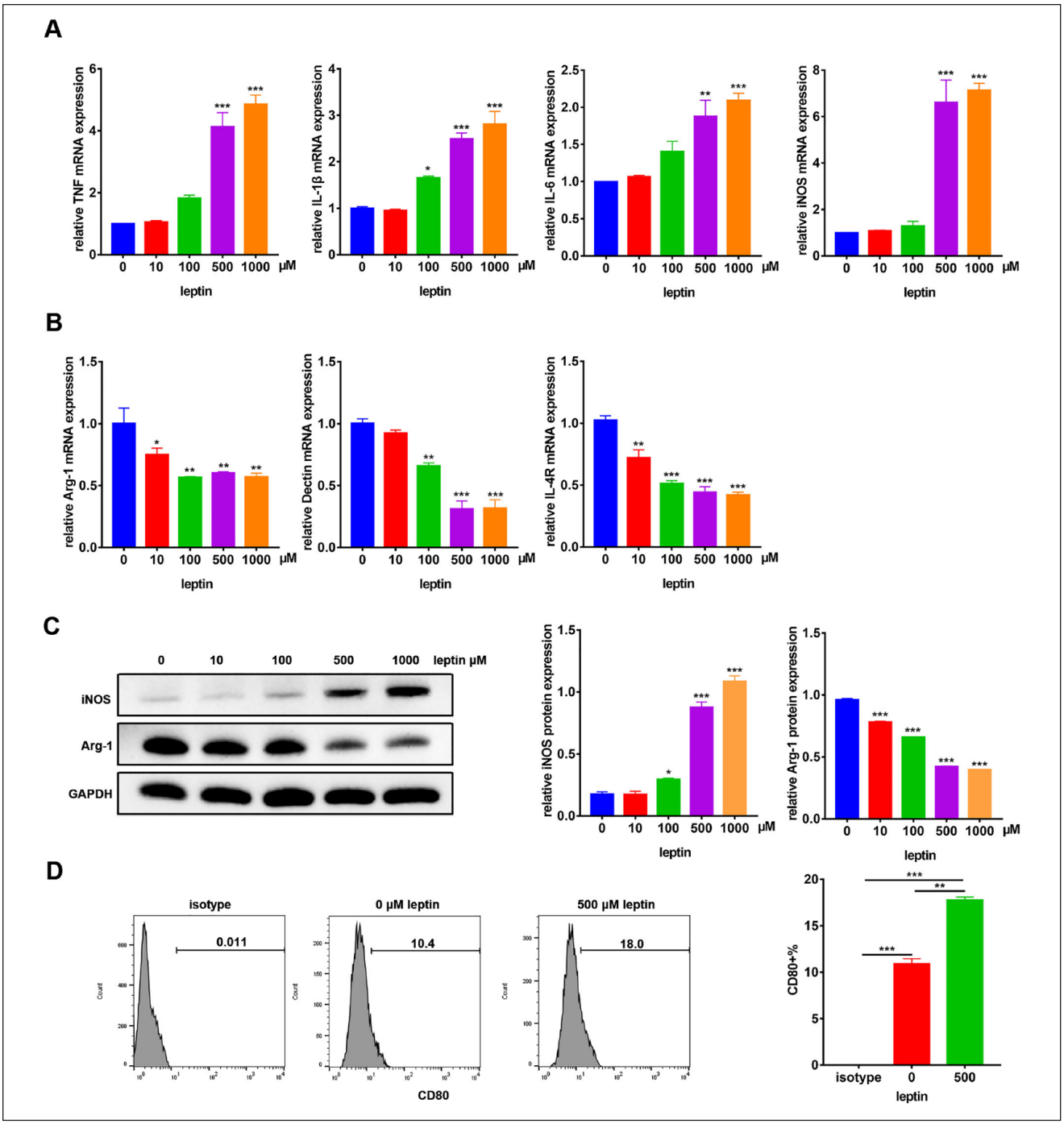

Figure 2. Leptin induces M1 macrophage polarization in vitro. (**A**) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) revealed the effects of leptin stimulation on M1 phenotype macrophage-associated genes, including tumor necrosis factor (TNF), interleukin (IL)–1β, IL-6, and inducible nitric oxide synthase (iNOS). (**B**) qRT-PCR revealed the effects of leptin stimulation on M2 phenotype macrophage-associated genes, including arginase 1 (Arg-1), dectin, and IL-4 receptor (IL-4R). (**C**) M1 marker iNOS and M2 marker Arg-1 were detected by Western blotting after leptin stimulation. The histograms show the quantification of band intensities. GAPDH was used for normalization. (**D**) Flow cytometry staining of RAW 264.7 cells for CD80-FITC. The percentage of positive cells for CD80 was determined. *$P<0.05$. **$P<0.01$. ***$P<0.001$.

Glogauer 2013; Lam et al. 2014; Gonzalez et al. 2015). In the present study, we observed more M1 phenotype macrophage infiltration in periodontal tissues and higher expression of serum leptin in periodontitis mice than in healthy controls.

At present, the role of leptin in macrophage function remains unclear. Both protective and harmful effects of leptin have been reported in various mouse models (Naylor and Petri 2016). One study reported that leptin stimulates IL-18 secretion in M2 macrophages to promote invasion and migration of

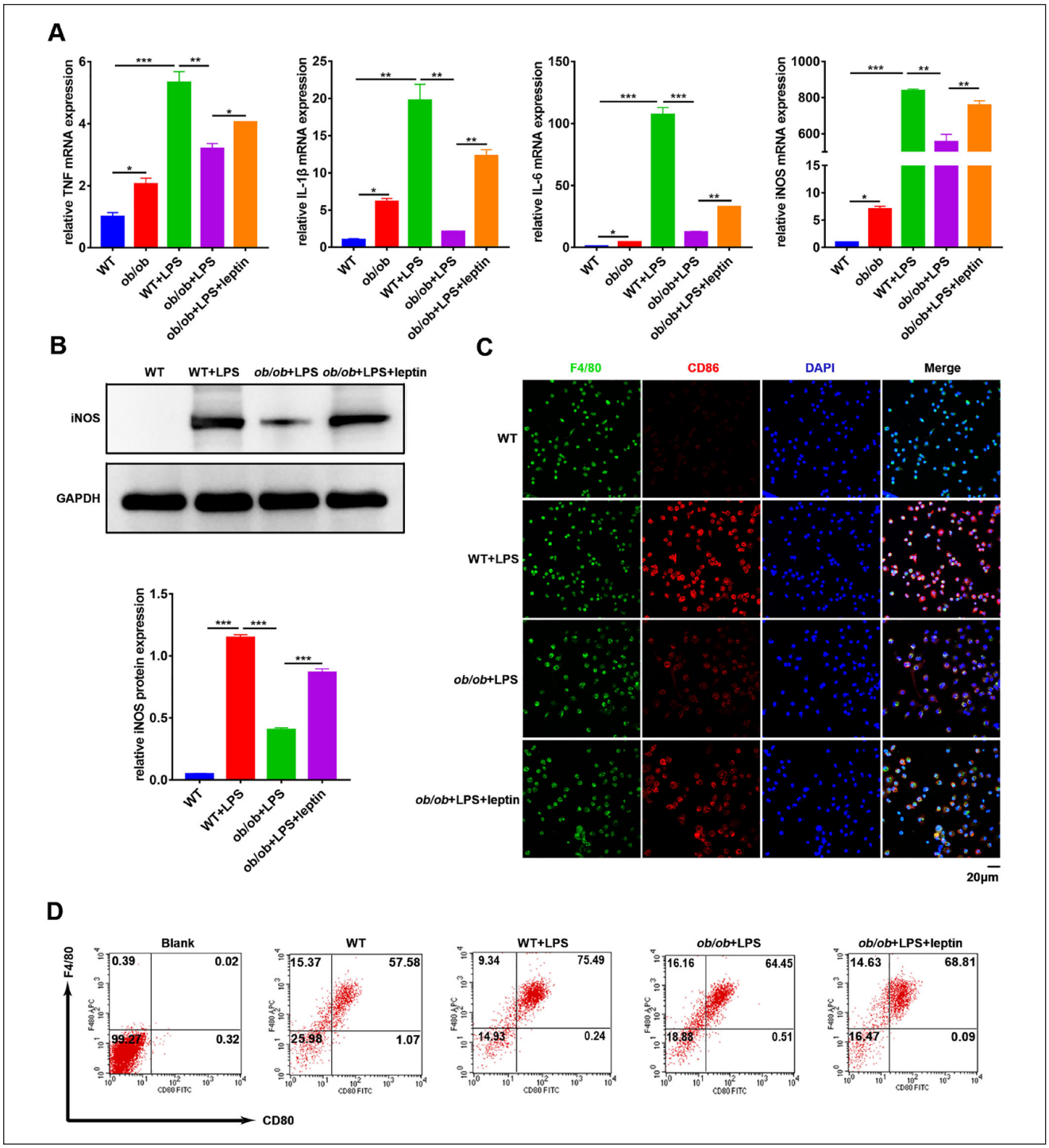

Figure 3. Leptin facilitates lipopolysaccharide (LPS)–induced M1 macrophage polarization in vitro. (**A**) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) revealed the expression of M1 markers, including interleukin (IL)–1β, IL-6, inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF) in bone marrow–derived macrophages (BMDMs) generated from WT and *ob/ob* mice. (**B**) M1 marker iNOS was detected by Western blotting in BMDMs generated from WT and *ob/ob* mice. The histogram shows the quantification of band intensities. GAPDH was used for normalization. (**C**) Immunofluorescence staining of F4/80 and CD86 in BMDMs generated from WT and *ob/ob* mice after LPS stimulation (scale bar = 20 μm). (**D**) Flow cytometry staining of BMDMs for F4/80-APC and CD80-FITC. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

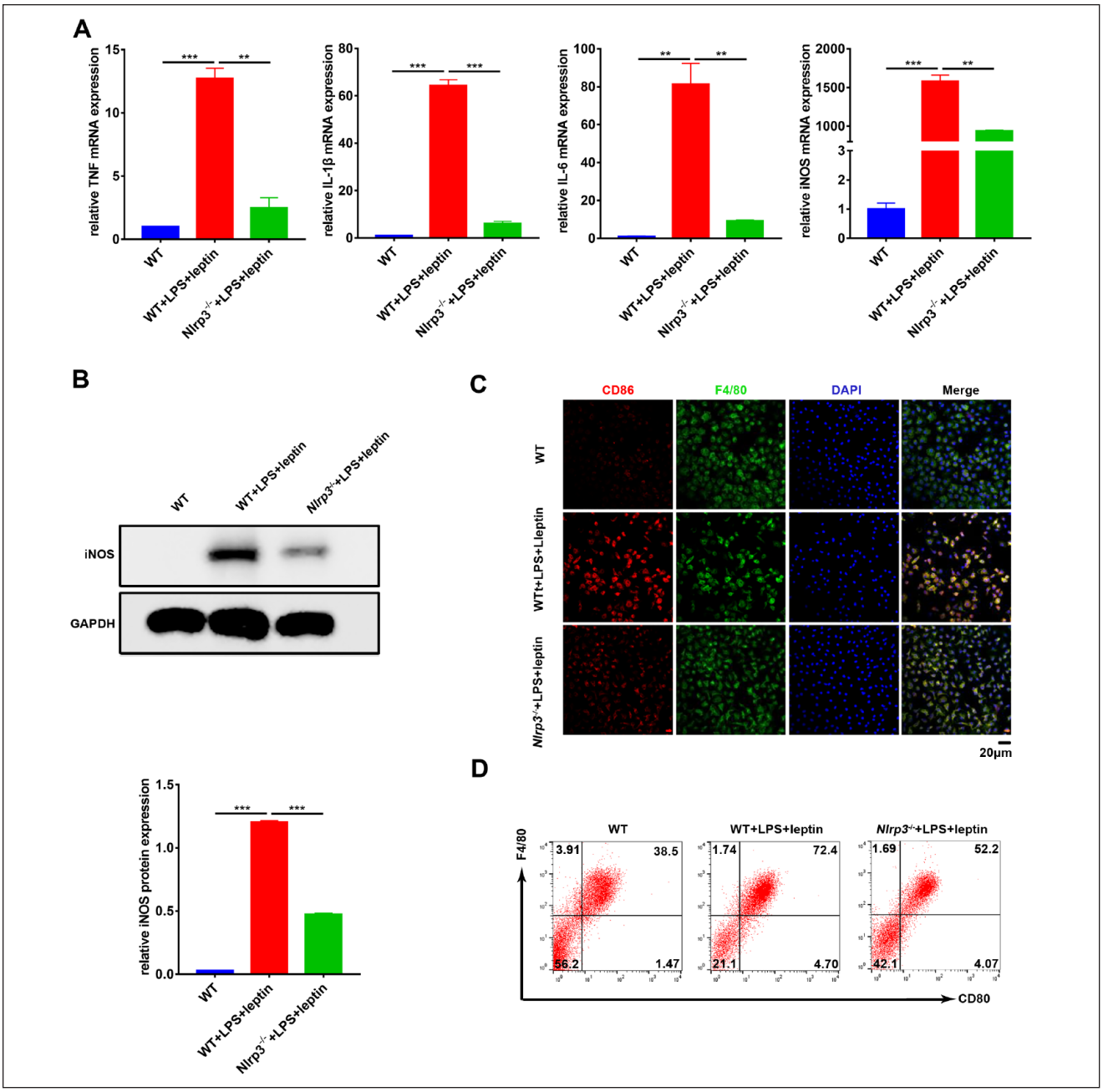

Figure 4. Leptin promotes M1 polarization via NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in vitro. (**A**) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) revealed the expression of M1 markers, including interleukin (IL)–1β, IL-6, inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF) in bone marrow–derived macrophages (BMDMs) generated from wild-type (WT) and *Nlrp3*$^{-/-}$ mice. (**B**) M1 marker iNOS was detected by Western blotting in BMDMs generated from WT and *Nlrp3*$^{-/-}$ mice after lipopolysaccharide (LPS) and leptin stimulation. The histogram shows the quantification of band intensities. GAPDH was used for normalization. (**C**) Immunofluorescence staining of F4/80 and CD86 in BMDMs generated from WT and *ob/ob* mice after LPS and leptin stimulation (scale bar = 20 μm). (**D**) Flow cytometry staining of BMDMs for F4/80-APC and CD80-FITC. $^{**}P < 0.01$. $^{***}P < 0.001$.

breast cancer cells (Cao et al. 2016). However, the effects of leptin on macrophage polarization were largely unknown. We found that leptin alone induced a low level of M1 polarization. When macrophages were treated with a combination of leptin and LPS, the polarization of M1 macrophages significantly increased and knockout of leptin markedly decreased M1 macrophage polarization in BMDMs generated from *ob/ob* mice after LPS stimulation. These results confirmed the proinflammatory role of leptin in macrophage polarization.

NLRP3 inflammasome is comprised of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and pro–caspase 1 (Yang et al. 1998). It can

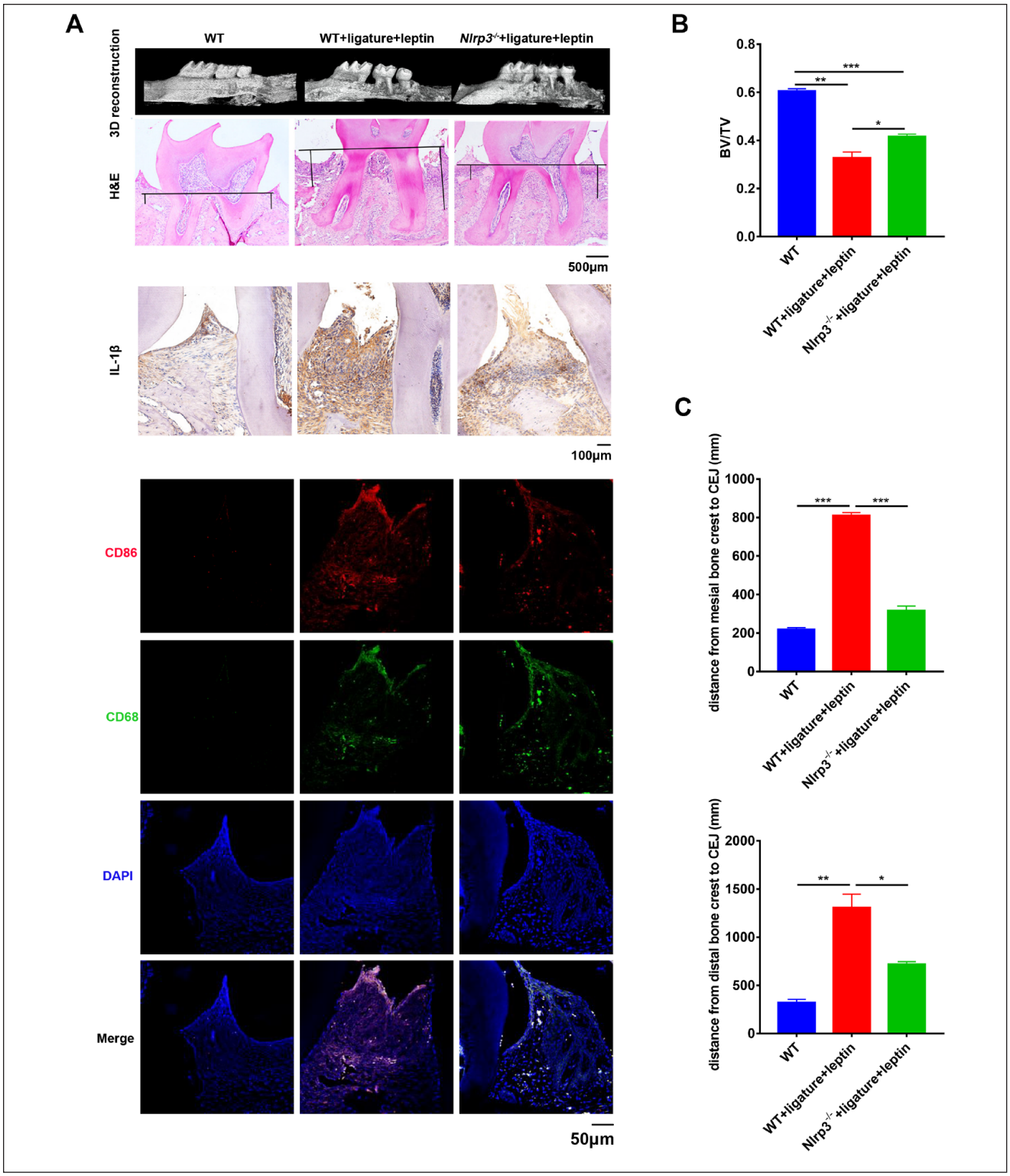

Figure 5. Leptin aggravates periodontitis progression via NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome-mediated M1 polarization in vivo. (**A**) Reconstructed 3-dimensional (3D) micro–computed tomography (CT) images of wild-type (WT), WT + ligature + leptin, and *Nlrp3*$^{-/-}$ + ligature + leptin groups. Hematoxylin and eosin staining (scale bar = 500 μm), immunohistochemistry staining (scale bar = 100 μm) of interleukin (IL)–1β, and immunofluorescence staining (scale bar = 50 μm) showing colocalization of CD86 (M1)–positive cells with infiltrated macrophage (CD68^{+}) in the gingival tissues of WT, WT + ligature + leptin, and *Nlrp3*$^{-/-}$ + ligature + leptin groups ($n = 5$/group). (**B**) Bone volume/tissue volume (BV/TV) analysis of WT, WT + ligature + leptin, and *Nlrp3*$^{-/-}$ + ligature + leptin groups. (**C**) The distances of the mesial and distal crest bone to the CEJ of WT, WT + ligature + leptin, and *Nlrp3*$^{-/-}$ + ligature + leptin groups. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

sense pathogens or sterile damage and subsequently activate IL-1β and IL-18 to mediate innate immune response (Liu et al. 2020). The activation of NLRP3 inflammasome depends on 2 signals: an initial priming signal 1, inducing the expression of pro–IL-1β, pro–IL-18, and NLRP3, and an activation signal 2, inducing cleavage to form the mature IL-1β and IL-18 (Mangan et al. 2018). Studies have reported that leptin activates the NLRP3 inflammasome and thus promotes the activation of IL-1β and IL-18 (Fu et al. 2017; Lin et al. 2020). In line with previous studies, our results confirmed that leptin activated NLRP3 in RAW 264.7 cells. In BMDMs generated from *ob/ob* mice, NLRP3 inflammasome activation was significantly reduced after LPS and ATP stimulation compared to the WT BMDMs. Raut et al. (2019) reported that leptin-induced NLRP3 inflammasome activation is mediated via estrogen receptor signaling and NADPH oxidase-derived reactive oxygen species production. However, the mediation of NLRP3 inflammasome activation by leptin is a complicated process, and whether leptin activates the NLRP3 inflammasome through other priming signal 1 and activation signal 2 needs further exploration.

Recently, the NLRP3 inflammasome has been suggested to be a key regulator of macrophage-induced inflammation (Zhang et al. 2020). Inhibition of the NLRP3 inflammasome reversed choline-metabolized trimethylamine N-oxide–stimulated M1 features (Wu, Yuan, et al. 2020). NLRP3 inflammasome also mediates M1 macrophage polarization in inflammatory root resorption during orthodontic tooth movement (Zhang et al. 2020). In the present study, we found that leptin aggravates periodontitis progression via NLRP3 inflammasome-mediated M1 polarization, and *Nlrp3* knockout protected against periodontal bone loss, inflammatory cytokine expression, and M1 macrophage infiltration. The limitation of our study was that we focused only on the effects of leptin on M1 macrophage polarization, and whether leptin influences M2 polarization requires further investigation.

In conclusion, our study demonstrated that elevated serum leptin expression during periodontitis promotes M1 macrophage polarization by activating NLRP3 inflammasome signaling. These activated macrophages secrete a large number of inflammatory factors, such as TNF and IL-1β, and then recruit more macrophages into the inflammation sites, destroy the periodontium, and ultimately lead to accelerated periodontitis progression. In the future, we will focus on how to regulate the expression of leptin in the serum, to develop a new strategy for the treatment of periodontitis.

Author Contributions

Y. Han, contributed to conception, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Y. Huang, contributed to conception, data analysis, and interpretation, critically revised the manuscript; P. Gao, Q. Yang, L. Jia, contributed to data analysis and interpretation, critically revised the manuscript; Y. Zheng, W. Li, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

We thank Xiaowei Wu from Peking University for his support in flow cytometric analysis.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was financially supported by grants from the National Natural Science Foundation of China (Nos. 82071119, 82071142).

References

Abella V, Scotece M, Conde J, Pino J, Gonzalez-Gay MA, Gomez-Reino JJ, Mera A, Lago F, Gomez R, Gualillo O. 2017. Leptin in the interplay of inflammation, metabolism and immune system disorders. Nat Rev Rheumatol. 13(2):100–109.

Acedo SC, Gambero S, Cunha FG, Lorand-Metze I, Gambero A. 2013. Participation of leptin in the determination of the macrophage phenotype: an additional role in adipocyte and macrophage crosstalk. In Vitro Cell Dev Biol Anim. 49(6):473–478.

Ahuja CR, Kolte AP, Kolte RA, Gupta M, Chari S. 2019. Effect of non-surgical periodontal treatment on gingival crevicular fluid and serum leptin levels in periodontally healthy chronic periodontitis and chronic periodontitis patients with type 2 diabetes mellitus. J Investig Clin Dent. 10(3):e12420.

Armitage GC; Research, Science and Therapy Committee of the American Academy of Periodontology. 2003. Diagnosis of periodontal diseases. J Periodontol. 74(8):1237–1247.

Cao H, Huang Y, Wang L, Wang H, Pang X, Li K, Dang W, Tang H, Wei L, Su M, et al. 2016. Leptin promotes migration and invasion of breast cancer cells by stimulating IL-8 production in M2 macrophages. Oncotarget. 7(40):65441–65453.

Conde J, Scotece M, Gomez R, Gomez-Reino JJ, Lago F, Gualillo O. 2010. At the crossroad between immunity and metabolism: focus on leptin. Expert Rev Clin Immunol. 6(5):801–808.

Fu S, Liu L, Han L, Yu Y. 2017. Leptin promotes IL18 secretion by activating the NLRP3 inflammasome in RAW 264.7 cells. Mol Med Rep. 16(6):9770–9776.

Gangadhar V, Ramesh A, Thomas B. 2011. Correlation between leptin and the health of the gingiva: a predictor of medical risk. Indian J Dent Res. 22(4):537–541.

Gonzalez OA, Novak MJ, Kirakodu S, Stromberg A, Nagarajan R, Huang CB, Chen KC, Orraca L, Martinez-Gonzalez J, Ebersole JL. 2015. Differential gene expression profiles reflecting macrophage polarization in aging and periodontitis gingival tissues. Immunol Invest. 44(7):643–664.

Han Y, Yang Q, Huang Y, Li X, Zhu Y, Jia L, Zheng Y, Li W. 2021. Mechanical force inhibited hPDLSCs proliferation with the downregulation of MIR31HG via DNA methylation. Oral Dis. 27(5):1268–1282.

Hou Y, Yu H, Liu X, Li G, Pan J, Zheng C, Yu W. 2017. Gingipain of *Porphyromonas gingivalis* manipulates M1 macrophage polarization through C5a pathway. In Vitro Cell Dev Biol Anim. 53(7):593–603.

Hsieh CC, Chou MJ, Wang CH. 2017. Lunasin attenuates obesity-related inflammation in RAW264.7 cells and 3T3-L1 adipocytes by inhibiting inflammatory cytokine production. PLoS One. 12(2):e0171969.

Huang X, Yu T, Ma C, Wang Y, Xie B, Xuan D, Zhang J. 2016. Macrophages play a key role in the obesity-induced periodontal innate immune dysfunction via nucleotide-binding oligomerization domain-like receptor protein 3 pathway. J Periodontol. 87(10):1195–1205.

Karthikeyan BV, Pradeep AR. 2007. Gingival crevicular fluid and serum leptin: their relationship to periodontal health and disease. J Clin Periodontol. 34(6):467–472.

La Cava A. 2017. Leptin in inflammation and autoimmunity. Cytokine. 98:51–58.

Lam RS, O'Brien-Simpson NM, Lenzo JC, Holden JA, Brammar GC, Walsh KA, McNaughtan JE, Rowler DK, Van Rooijen N, Reynolds EC. 2014. Macrophage depletion abates *Porphyromonas gingivalis*–induced alveolar bone resorption in mice. J Immunol. 193(5):2349–2362.

Lin N, Song X, Chen B, Ye H, Wang Y, Cheng X, Wang H. 2020. Leptin is upregulated in epididymitis and promotes apoptosis and IL-1β production

in epididymal epithelial cells by activating the NLRP3 inflammasome. Int Immunopharmacol. 88:106901.
Liu D, Zeng X, Li X, Cui C, Hou R, Guo Z, Mehta JL, Wang X. 2020. Advances in the molecular mechanisms of NLRP3 inflammasome activators and inactivators. Biochem Pharmacol. 175:113863.
Liu YY, Zhang X, Shi D, Cheng ZB, Meng HX. 2013. Association between plasma leptin level and periodontal parameters in patients with aggressive periodontitis. Zhonghua Kou Qiang Yi Xue Za Zhi. 48(1):3–6.
Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E. 2018. Targeting the NLRP3 inflammasome in inflammatory diseases. Nat Rev Drug Discov. 17(9):688.
Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25(12):677–686.
Naylor C, Petri WA Jr. 2016. Leptin regulation of immune responses. Trends Mol Med. 22(2):88–98.
Philbrick KA, Branscum AJ, Wong CP, Turner RT, Iwaniec UT. 2018. Leptin increases particle-induced osteolysis in female ob/ob mice. Sci Rep. 8(1):14790.
Raut PK, Kim SH, Choi DY, Jeong GS, Park PH. 2019. Growth of breast cancer cells by leptin is mediated via activation of the inflammasome: critical roles of estrogen receptor signaling and reactive oxygen species production. Biochem Pharmacol. 161:73–88.
Seymour GJ, Gemmell E, Reinhardt RA, Eastcott J, Taubman MA. 1993. Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. J Periodontal Res. 28(6 Pt 2): 478–486.
Shimada Y, Komatsu Y, Ikezawa-Suzuki I, Tai H, Sugita N, Yoshie H. 2010. The effect of periodontal treatment on serum leptin, interleukin-6, and C-reactive protein. J Periodontol. 81(8):1118–1123.
Sima C, Glogauer M. 2013. Macrophage subsets and osteoimmunology: tuning of the immunological recognition and effector systems that maintain alveolar bone. Periodontol 2000. 63(1):80–101.
Singh P, Peterson TE, Barber KR, Kuniyoshi FS, Jensen A, Hoffmann M, Shamsuzzaman AS, Somers VK. 2010. Leptin upregulates the expression of plasminogen activator inhibitor-1 in human vascular endothelial cells. Biochem Biophys Res Commun. 392(1):47–52.
Su L, Zhang W, Wu X, Zhang Y, Chen X, Liu G, Chen G, Jiang M. 2015. Glycocalyx-mimicking nanoparticles for stimulation and polarization of macrophages via specific interactions. Small. 11(33):4191–4200.
Talari M, Kapadia B, Kain V, Seshadri S, Prajapati B, Rajput P, Misra P, Parsa KV. 2015. MicroRNA-16 modulates macrophage polarization leading to improved insulin sensitivity in myoblasts. Biochimie. 119:16–26.
Wu K, Yuan Y, Yu H, Dai X, Wang S, Sun Z, Wang F, Fei H, Lin Q, Jiang H, et al. 2020. The gut microbial metabolite trimethylamine N-oxide aggravates GVHD by inducing M1 macrophage polarization in mice. Blood. 136(4):501–515.
Wu X, Chen H, Wang Y, Gu Y. 2020. Akt2 affects periodontal inflammation via altering the M1/M2 ratio. J Dent Res. 99(5):577–587.
Yan B, Liu D, Zhang C, Zhang T, Wang X, Yang R, Liu Y, He D, Zhou Y. 2018. Obesity attenuates force-induced tooth movement in mice with the elevation of leptin level: a preliminary translational study. Am J Transl Res. 10(12):4107–4118.
Yang J, Zhu Y, Duan D, Wang P, Xin Y, Bai L, Liu Y, Xu Y. 2018. Enhanced activity of macrophage M1/M2 phenotypes in periodontitis. Arch Oral Biol. 96:234–242.
Yang X, Chang HY, Baltimore D. 1998. Autoproteolytic activation of procaspases by oligomerization. Mol Cell. 1(2):319–325.
Yu Y, Fu S, Zhang X, Wang L, Zhao L, Wan W, Xue Y, Lv L. 2020. Leptin facilitates the differentiation of Th17 cells from MRL/Mp-Fas lpr lupus mice by activating NLRP3 inflammasome. Innate Immun. 26(4):294–300.
Zhang J, Liu X, Wan C, Liu Y, Wang Y, Meng C, Zhang Y, Jiang C. 2020. NLRP3 inflammasome mediates M1 macrophage polarization and IL-1β production in inflammatory root resorption. J Clin Periodontol. 47(4):451–460.
Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature. 372(6505):425–432.
Zhou Y, Yu X, Chen H, Sjoberg S, Roux J, Zhang L, Ivoulsou AH, Bensaid F, Liu CL, Liu J, et al. 2015. Leptin deficiency shifts mast cells toward anti-inflammatory actions and protects mice from obesity and diabetes by polarizing M2 macrophages. Cell Metab. 22(6):1045–1058.
Zimmermann GS, Bastos MF, Dias Goncalves TE, Chambrone L, Duarte PM. 2013. Local and circulating levels of adipocytokines in obese and normal weight individuals with chronic periodontitis. J Periodontol. 84(5):624–633.