

All-*Trans* Retinoic Acid Modulates The Immune Status of M2 Macrophages in Experimental Periodontitis Induced by *Porphyromonas Gingivalis* in Mice

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Abstract

Background: M2 macrophages are important innate immune cells that participate in the pathogenesis of periodontitis. The effect of all-trans retinoic acid (ATRA) on the immune status of M2 macrophages in periodontitis has not been reported.

Methods: An experimental model of periodontitis was established in mice by oral administration of *Porphyromonas gingivalis*, and then ATRA or vehicle was administered orally to model mice every other day (P.g+ATRA and P.g+CMC mice, respectively). Flow cytometry was used to analyze the numbers of F4/80+CD206+ M2 macrophages in the gingiva, spleen, and peritoneal lavage fluid (PLF). M2 macrophage-related cytokines were quantified by real-time reverse transcription-polymerase chain reaction.

Results: Compared with P.g+CMC mice, P.g+ATRA mice showed a significantly reduced cemento-enamel junction to alveolar bone crest (CEJ-ABC) distance. The percentage of F4/80+CD206+ M2 macrophages in gingiva, PLF and spleen in model mice increased after ATRA treatment. The mRNA expression levels of M2 macrophage-related cytokines (IL-10, TGF- β 1 and Arg-1) in gingiva, PLF and spleen of P.g+ATRA mice were higher than those of P.g+CMC mice.

Conclusions: These results suggested that ATRA modulates the immune status of M2 macrophages and provides protection against periodontitis by enhancing M2 activation.

Introduction

Periodontitis is a disease involving the destruction of periodontal supporting tissue caused by unattached subgingival plaque and its metabolites in the gingival sulcus. Periodontitis is characterized by the loss of attachment, alveolar bone resorption, and periodontal pocket formation. The host immune response, including both innate and adaptive immune responses, is initiated against periodontal pathogens and plays an important role in the occurrence, development, and prognosis of periodontitis[1]. Resident macrophages in gingival tissue secrete large numbers of proinflammatory cytokines and chemokines after interactions with periodontal microorganisms. The macrophages participate in the phagocytosis and inflammation of periodontal pathogenic microorganisms and regulate alveolar bone resorption [2].

Macrophages are heterogeneous cells with strong plasticity and functional diversity that play key roles in both innate and adaptive immunity. Macrophages can be divided into many types based on their activated phenotypes and functions, including the classically and alternatively activated macrophages, designated as M1 and M2 macrophages, respectively. The phenotypic variations in macrophages induced by activation are reversible. M2 macrophages are activated by Th2 cytokines, such as interleukin (IL)-4, IL-13, and IL-10 [3,4], and secrete anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonists, as well as high levels of mannose receptor (CD206), arginase-1 (Arg-1), chitinase-3-like protein 1 (CHI3L1), and resistin-like molecule alpha (RELM α). M2 macrophages play important roles in the repair and remodeling of tissue damage caused by pathogenic microorganisms in the later stages of

inflammation [5,6]. Intraperitoneal injection of M2 macrophages was reported to reduce experimental colitis in mice via an IL-10-dependent mechanism [7]. In a study in an experimental periodontitis model, adiponectin was suggested to participate in M2 macrophage polarization and host response by regulating the JMJD3-IRF4 axis, thus alleviating alveolar bone resorption [8]. Therefore, regulation of macrophage polarization to M2 macrophages may represent a new strategy for the treatment of periodontitis.

All-*trans* retinoic acid (ATRA) is an active metabolite of vitamin A that has a wide range of immunomodulatory effects and plays important roles in immune cell differentiation and activation as well as maintenance of the immune system. Studies showed that ATRA can be used in the treatment of a wide range of autoimmune diseases [9]. ATRA also regulates dendritic cells and reduces their antigen presentation ability, which further stimulate the T cells to respond to Th2 [10]. In addition, the combination of ATRA and IL-4 significantly increased the expression of arginase-1 (Arg-1), which is a marker of M2 activation [11]. However, there have been no studies on the effects of ATRA on the immune status of M2 macrophages in periodontitis. In this study, we examined the effects of ATRA on the immune status of M2 macrophages in periodontitis using a mouse model of periodontitis.

Materials And Methods

Mice

The experiments were performed in 7-week-old specific pathogen free C57BL/6 female mice weighing ~18 g, with intact dentition and no caries or periodontal disease. Mice were obtained from the Animal Center of Jinzhou Medical University. Animals were kept in individual cages with ad libitum access to food and water and fed adaptively for 1 week before establishment of the periodontitis model. The experimental procedures and animal treatment protocols were approved by the Ethics Committee of Jinzhou Medical University.

Mouse periodontitis model

Porphyromonas gingivalis W83 was cultivated on brain heart infusion (BHI) blood agar medium (Solarbio, Beijing, China) (containing 5% sheep's blood (Solarbio), 1% hemin (Sigma-Aldrich, MO, USA), and 0.1% vitamin K1 (Huayang, Jiangsu, China)) for 5–7 days. Colonies were scraped off the plates and re-suspended in BHI liquid culture medium. *P. gingivalis* W83 was collected after 24 h. The bacterial density was adjusted to 1×10^9 /mL in phosphate buffer containing 2% sodium carboxy methylcellulose (CMC) (Macklin, Shanghai, China).

Fifteen mice were randomly divided into three groups: Sham, *P.g*+ATRA, and *P.g*+CMC (n=5 mice/group). Periodontitis model was induced in mice by oral infection with 10^9 *P. gingivalis* W83, suspended in 100 μ l sterile phosphate-buffered saline (PBS) containing 2% CMC, twice a day for 7 days. Sham mice were

administered 100 µl of PBS containing 2% CMC by a micropipette for 7 days. Mice in the *P.g*+ATRA group were intra-gastrically administered 200 µL of ATRA per mouse dissolved in 1% CMC solution at 0.25 mg/µl every other day from day 0 to day 42, and mice in the *P.g*+CMC and Sham group were intra-gastrically administered 200 µl of 1%CMC solution per mouse. All mice were killed by asphyxiation with CO₂ on day 42 and subjected to subsequent analyses described below.

Bone loss measurement

The right maxilla was dissected out and the soft tissue was removed by soaking in 3% hydrogen peroxide for 6 h, followed by boiling for 5 min to remove the residual soft tissue; the tissues were then stained with 0.1% methylene blue. The distance from the cement enamel junction (CEJ) of the maxillary first molar to the alveolar bone crest (ABC) of the third molar (CEJ-ABC distance) was measured under a stereomicroscope at 32× magnification. The mesial as well as the buccal and palatal molar surfaces were measured in each tooth, and the average values of 12 sites were determined. The total CEJ-ABC distance of the 12 sites in each group was calculated. The bone resorption rate of the experimental group (%) was calculated as = (total CEJ-ABC distance of the experimental group – total CEJ-ABC distance of the Sham group) / total CEJ-ABC distance of the Sham group.

Histological examination

Staining with hematoxylin and eosin (H&E) was performed to visualize the pathological damage of periodontal tissue. The molar segment of the left maxilla was washed with phosphate buffer, fixed with 4% paraformaldehyde for 48 h, and decalcified in 10% neutral ethylenediamine tetra acetic acid (EDTA; Solarbio). After dehydration in an alcohol series and paraffin embedding, the samples were cut into proximal and distal sections (5 µm thick). The sections were then stained with H&E and examined under a dissecting microscope at 100× magnification.

Flow cytometric analysis

Isolation of gingival mononuclear cells

The isolated periodontal tissue was washed with PBS and then added to 1 mL of PBS containing 2% FCS, 2 mg/mL collagenase II (Invitrogen, CA, USA), 1 mg/mL DNase I (Solarbio), and 1 mg/mL hyaluronidase (Sigma-Aldrich) in centrifuge tubes. Next, 20 µL of 0.5 M EDTA was added, and the tubes were incubated horizontally in a water bath preheated at 37°C with shaking at 200 rpm for 60 min. After centrifugation at 400 xg, 4°C for 8 min, the supernatant was discarded and the pellet was passed through a 200 mesh sieve. The filtered cell suspension was then centrifuged at 320 xg, 4°C for 8 min, and the supernatant was

discarded. The pellet was resuspended in 200 μ L of 2% fetal bovine serum (FBS) in PBS (FBS-PBS) and the cell numbers were counted.

Extraction of peritoneal lavage fluid

Under aseptic conditions, the abdominal cavity of mice was opened and injected with 5 mL of precooled 2% FBS-PBS gently over a period of 2–3 min. After holding for an additional 5 min, the peritoneal lavage fluid was absorbed and lavaged again. The fluid was collected into 15-mL centrifuge tubes and the tubes were centrifuged at $300 \times g$, 4°C for 8 min. The supernatant was discarded; the pellet was resuspended in 200 μ L of 2% FBS-PBS and the cell numbers were counted.

Preparation of single cell suspensions of spleen cells

The spleens of mice were removed aseptically according to routine procedures, rinsed with 1 mL of PBS, ground on a 200-mesh stainless steel screen, and filtered through a 30- μ m nylon mesh to remove agglomerated cells. The cell suspension was centrifuged at 300 \times g, 4°C for 5 min, and then the supernatant was aspirated. Next, 1 mL red blood cell lysate was added to the precipitated cell mass. RPMI1640 medium containing 10% FBS and 1% penicillin-streptomycin was then added to terminate the reaction. After centrifugation at 300 \times g, 4°C for 5 min, the supernatant was discarded. The pellet was then washed twice with PBS and the supernatant was discarded. Cell viability was determined by trypan blue staining, and the cells were counted.

To identify macrophages and macrophage subpopulations, cells were stained with FITC-labeled monoclonal anti-mouse F4/80 and APC-labeled monoclonal anti-mouse CD206 antibodies (BioLegend, San Diego, CA, USA). The cells were washed twice with PBS containing 1% FBS, resuspended in staining buffer, and examined by flow cytometry (BD FACSAria II, NY, USA). F4/80⁺CD206⁺ cells (M2 macrophages) were analyzed using Flow Jo10.4 software.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For qRT-PCR, total RNA was extracted from gingival tissues, peritoneal cells, and splenocytes using TRIzol reagent (Vazyme Biotechnology Co. Ltd, Nanjing, China) in accordance with the manufacturer's protocol. The quality and quantity of RNA were measured using a spectrophotometer. The absorbance was measured at wavelengths of 260 nm and 280 nm and the ratios of the optical densities (ODs) at 260/280 nm for the samples were between 2.0 and 2.2. Total RNA was reverse transcribed into cDNA for amplification using reverse transcription (RT) reaction (PrimeScript RT system; Vazyme Biotechnology Co. Ltd.) according to the manufacturer's instructions. PCR was performed in an ABI PRISM cycler with the SYBR Green PCR Kit and specific primers for IL-10, Arg-1, TGF- β 1, and b-actin mRNAs. PCR was

performed in a total volume of 20 μ L consisting of 10 μ L of 2 \times ChamQuniversal SYBR qPCR Master Mix, 0.4 μ L each of forward and reverse primers, 2 μ L of cDNA, and ddH₂O.

The primer sequences are as follows:

mouse IL-10, forward, 5'-CCTCGTTTGTACCTCTCTCCG-3';

mouse IL-10, reverse, 5'-AGGACACCATAGCAAAGGGC-3';

mouse TGF- β 1, forward, 5'-AGCTGCGCTTGACAGAGATTA-3';

mouse TGF- β 1, reverse, 5'-AGCCCTGTATTCCGTCTCCT-3';

mouse Arg-1, forward, 5'-CAGCACTGAGGAAAGCTGGT-3';

mouse Arg-1, reverse, 5'-ACAGACCGTGGGTTCTTCAC-3';

mouse b-actin, forward, 5'-GGCTGTATTCCCCTCCATCG-3';

mouse b-actin, reverse, 5'-CCAGTTGGTAACAATGCCATGT-3'.

The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression level of each gene. The target gene mRNA expression was normalized relative to b-actin mRNA, and the adjusted gene expression of mice in the Sham group was used as a reference (fold change = 1).

Statistical analysis

The data are expressed as the mean \pm standard deviation (SD) and were analyzed using GraphPad Prism 22.0 software. The results were analyzed by one-way analysis of variance (ANOVA) and Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

ATRA prevented the body weight loss in mice induced by *P. gingivalis* infection

To examine the effects of ATRA on the mouse model of periodontitis established by infection with *P. gingivalis*, we first compared the changes in body weight between the *P.g*+ATRA, *P.g*+CMC, and Sham groups. The mice in the *P.g*+CMC group weighed significantly less than those in the Sham group on day 21 ($P < 0.01$, Figure 1). The body weights of the mice in the *P.g*+ATRA group began to show a significant upward trend on day 21 compared with mice in the *P.g*+CMC group, which continued until the animals were sacrificed on day 42 ($P < 0.01$, Figure 1). These data suggest that ATRA can improve the changes in body weight in periodontitis mice, possibly by ameliorating the effects of periodontitis.

ATRA reduced alveolar bone loss induced by *P. gingivalis* infection

To examine the effects of ATRA on periodontitis in mice infected with *P. gingivalis*, we measured the CEJ-ABC distance in the *P.g*+ATRA, *P.g*+CMC, and Sham groups. The CEJ-ABC distance was significantly increased in the *P.g*+CMC mice compared with the distance in the Sham mice ($P < 0.01$, Figure 2a, b). However, the CEJ-ABC distance in *P.g*+ATRA mice was greatly reduced compared with the distance in the *P.g*+CMC mice ($P < 0.01$). No significant difference was observed in alveolar bone resorption between the *P.g*+ATRA mice and Sham mice ($P < 0.05$, Figure 2b).

The alveolar bone resorption rates of both the Sham and the *P.g*+ATRA mice were significantly lower than that of the *P.g*+CMC mice ($0\% \pm 6.6\%$ and $16.2\% \pm 7.4\%$ vs. $54.3\% \pm 8.7\%$, respectively; all, $P < 0.01$, Figure 2c).

ATRA attenuated periodontal lesions induced by *P. gingivalis*

Histological analysis with H&E staining indicated that the epithelium in the sulcus was intact in the Sham mice; there was no inflammatory cell infiltration in gingival epithelium and the periodontal ligament fiber showed an orderly and regular arrangement (Figure 3). In contrast, the *P.g*+CMC mice showed inflammatory cell infiltration and collagen fiber degeneration and destruction. We also detected infiltration of large numbers of inflammatory cells and loss of attachment. ATRA treatment resulted in reduced inflammatory cell infiltration and attachment losses in *P. gingivalis*-infected periodontal tissues. Moreover, there was no apparent alveolar bone resorption in *P.g*+ATRA mice compared with that observed in *P.g*+CMC mice.

ATRA increased the proportion of M2 macrophages during *P. gingivalis* infection

To clarify the role of ATRA in the immune status of M2 macrophages in mouse periodontitis, the percentages of F4/80⁺CD206⁺ cells (M2 macrophages) in gingivae, peritoneal cavity, and spleen were analyzed by flow cytometry. The results showed that the proportions of F4/80⁺CD206⁺ M2 macrophages in gingival mononuclear cells, peritoneal lavage fluid (PLF), and spleen were significantly decreased in the *P.g*+CMC mice compared with levels in the Sham mice ($P < 0.01$, Figure 4). In contrast, the *P.g*+ATRA mice showed significantly higher proportions of F4/80⁺CD206⁺ M2 macrophages in gingival mononuclear cells, PLF, and spleen compared with levels in the *P.g*+CMC mice ($P < 0.01$, Figure 4).

ATRA upregulated the mRNA expression of M2 macrophage–related cytokines in periodontitis

qRT-PCR analyses were performed to determine the mRNA expression levels of M2 macrophage–related cytokines in gingivae, PLF, and spleen. The levels of IL-10 and TGF- β 1 mRNAs in gingivae and PLF were significantly increased in the *P.g*+CMC mice compared with levels in the Sham mice ($P < 0.01$, Figure 5a, b). Arg1 mRNA expression levels in gingival tissue were significantly higher in the *P.g*+CMC mice than in the Sham mice ($P < 0.01$, Figure 5a), but there were no significant differences in the PLF and spleen ($P > 0.05$, Figure 5b, c). The levels of IL-10, TGF- β 1, and Arg-1 mRNAs in the gingivae, PLF, and spleen were significantly higher in the *P.g*+ATRA mice than in the *P.g*+CMC mice ($P < 0.01$, Figure 5a–c).

Discussion

The clinical treatment of periodontitis currently includes plaque removal, surgical treatment, auxiliary laser, drug therapy, and other treatments. However, plaque removal and periodontal surgery have limited efficacy in the management of periodontitis [12]. Given the critical role of the immune system in the occurrence, development, and prognosis of periodontitis, effective regulation of the immune response is critical to enable it to play a protective role against periodontitis. Macrophages are involved in the development and persistence of inflammatory responses and the protection against periodontitis [13]. M2 macrophages are immunomodulatory cells that are characterized by the expressions of c-MAF, Arginase-1, CD14, CD163, mannose receptor (CD206), IL-10 and TGF- β 1 [14]. Our study results indicate that ATRA upregulated the immune status of M2 macrophages in a mouse model of periodontitis and effectively inhibited periodontal tissue destruction. ATRA increased the proportions of F4/80⁺CD206⁺ M2 macrophages in gingival tissue, spleen, and PLF and upregulated the expression of IL-10, TGF- β 1, and Arg-1 mRNAs, which may have a protective effect against periodontitis.

Some studies have shown that *P. gingivalis* inhibits the production of α -ketoglutaric acid by M2 macrophages, thus maintaining the high inflammatory state of the periodontal microenvironment [15]. Miao et al.[16] reported that the proportion of M2 macrophages was significantly smaller in a mouse model of periodontitis induced by *P. gingivalis* compared with the healthy control group. These results imply that M2 macrophages are involved in maintaining periodontal tissue homeostasis in the periodontal microenvironment. Consistent with the above results, we found that the proportion of M2 macrophages in the gingival tissue of mice with periodontitis decreased in the model mice compared with the Sham group, while the mRNA expression levels of M2 macrophage–related cytokines IL-10, TGF- β 1, and Arg1 increased. Although the levels of anti-inflammatory factors increased in mice with periodontitis, periodontal attachment loss and alveolar crest resorption were observed, which may have been because the efficacy of the anti-inflammatory cytokines secreted by M2 macrophages was insufficient to resist the tissue destruction caused by inflammation with the progression of inflammatory periodontitis. These results indicate a weak macrophage-mediated immune response in periodontitis.

Fotinoset al.[17] reported that the autoimmune reaction caused by severe periodontitis resulted in the accumulation of large numbers of immune cells, such as macrophages, in the periodontal tissue that destroyed the basement membrane. Destruction of the basement membrane allowed toxic compounds such as lipopolysaccharide to penetrate across the gingival epithelial barrier and enter the bloodstream, resulting in a continuous stimulation of the systemic host response and increased levels of inflammation and C-reactive protein in the body. Consistent with the findings of Fotinos et al., in the present study, we detected the presence of M2 macrophages and the expression of M2 macrophage-related factors in the PLF and spleen of mice with periodontitis. The results were consistent with the changes in the proportion of M2 macrophages seen in gingival tissue. The proportion of M2 macrophages decreased in the PLF and spleen, but there were no significant changes in anti-inflammatory cytokine levels. These observations imply that the immune response mediated by M2 macrophages in the PLF and spleen is weakened in periodontitis.

Enhancement of the immune response mediated by M2 macrophages has been reported to ameliorate the inflammatory response and promote tissue repair. Hunter et al. [8] reported that adoptive infusion of M2 macrophages reduced the degree of inflammation in mice with colitis induced by dinitrobenzene sulfonic acid. In an animal model of experimental autoimmune encephalomyelitis, Liu et al. [18] reported that upregulation of the expressions of Arg-1, CD206, CD14, and IL-10 in the spleen induced the transformation of inflammatory M1 macrophages into anti-inflammatory M2 macrophages, thus increasing demyelination and inhibiting the progression of inflammation. Zhuang et al. [19] reported that the proportion of CD206⁺ M2 macrophages in gingival tissue increased, the number of osteoclasts decreased, and bone resorption decreased following injection of CCL2 into the gingivae of mice orally infected with *P. gingivalis*.

ATRA has a number of biological roles in cell proliferation and differentiation, embryogenesis, immunity, and metabolism [20]. ATRA was reported to induce the secretion of M2 macrophage chemokines in both C57BL/6 (B6) and BALB/c mice by inhibiting the expression of pro-inflammatory cytokines, nitric oxide synthase (NOS2), and NO and promoted the transition of macrophages from M1 to M2 in the two mouse strains [10]. Feng et al.[3] reported that ATRA reduced the expression of CD80, CD86 and CCR7 in splenic macrophages, increased the expression of CD209, promoted the polarization of macrophages to M2 macrophages, enhanced the production of IL-10 in M2 macrophages and inhibited the secretion of IL-12 and TNF- α , which further alleviated the progression of idiopathic thrombocytopenic purpura. Our previous studies confirmed that ATRA regulates the imbalance of Th17/Treg cells and improves periodontitis caused by *P. gingivalis*. These activities were likely mediated by ATRA inhibiting the response of Th17 cells, enhancing the response of Treg cells, and reducing the expression of RANKL in CD4⁺ T cells, thus regulating the Th17/Treg imbalance[21]. In the present study, oral administration of ATRA in a *P. gingivalis*-induced periodontitis mouse model resulted in an increase in F4/80⁺CD206⁺ M2 macrophages; there was no obvious alveolar bone resorption, and the mRNA expression levels of M2 macrophage-related factors IL-10, TGF- β 1, and Arg1 in gingival tissue increased. Similar results were

observed in the spleen and PLF. Therefore, ATRA not only modulates the immune state of M2 macrophages in the periodontal region, but also regulates the systemic immune state.

ATRA has been reported to activate JAK1/3 by regulating the binding of IL-4 with its receptor IL-4R. Phosphorylated JAK1/3 then recruits signal transduction and transcriptional activator 6 (STAT6) to promote the gene expression of Arg1, a key gene for tissue repair in M2 macrophages [22]. Lee et al. [23] reported that ATRA activates Arg-1 by upregulating the transcription of biosynthetic rate-limiting enzyme Raldh2, thereby promoting wound healing. ATRA was reported to induce the expression of TIMP1 in human alveolar M2 macrophages and regulate tissue remodeling by inhibiting the degradation of the extracellular matrix by various matrix metalloproteinases[24]. Retinoic acid nuclear receptor RAR-b is highly expressed in peritoneal macrophages, and retinoic acid response elements are also present in the gene regulatory regions of specific GATA6 transcription factors expressed in peritoneal macrophages. ATRA regulates the expression of Arg-1-specific genes in peritoneal macrophages through GATA6 transcription factors [25,26]. Taken together, the above results imply that ATRA affects the polarization of macrophages through a variety of signaling pathways.

In conclusion, the results of this study demonstrate that oral administration of ATRA modulated the immune status of M2 macrophages, affected the local oral microenvironment, and improved the inflammatory state in an experimental mouse model of *P. gingivalis*-induced periodontitis. Treatment with ATRA reduced both alveolar bone loss and the systemic immune response. These observations provide a basis for studying the influence of periodontitis on innate immune function, as well as the relationships between periodontitis, systemic health, and immune regulation of periodontitis.

Declarations

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Conflict of Interest statement

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Jinzhou Medical University .

Authors' contributions

WJZ and LYW conceived the study. WJZ drafted the manuscript. LYW revised the manuscript. Flow cytometry was completed by WLY. *P.gingivalis* W83 culture and mouse periodontitis model was completed by XQG and NG. Reverse transcription- quantitative PCR was performed by WJZ. All authors have read and approved the manuscript.

Consent to participate All authors listed in the article have approved the manuscript that is enclosed.

Consent to publish The manuscript is approved by all authors for publication.

Informed consent All authors agree and give consent for the publication.

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Figures

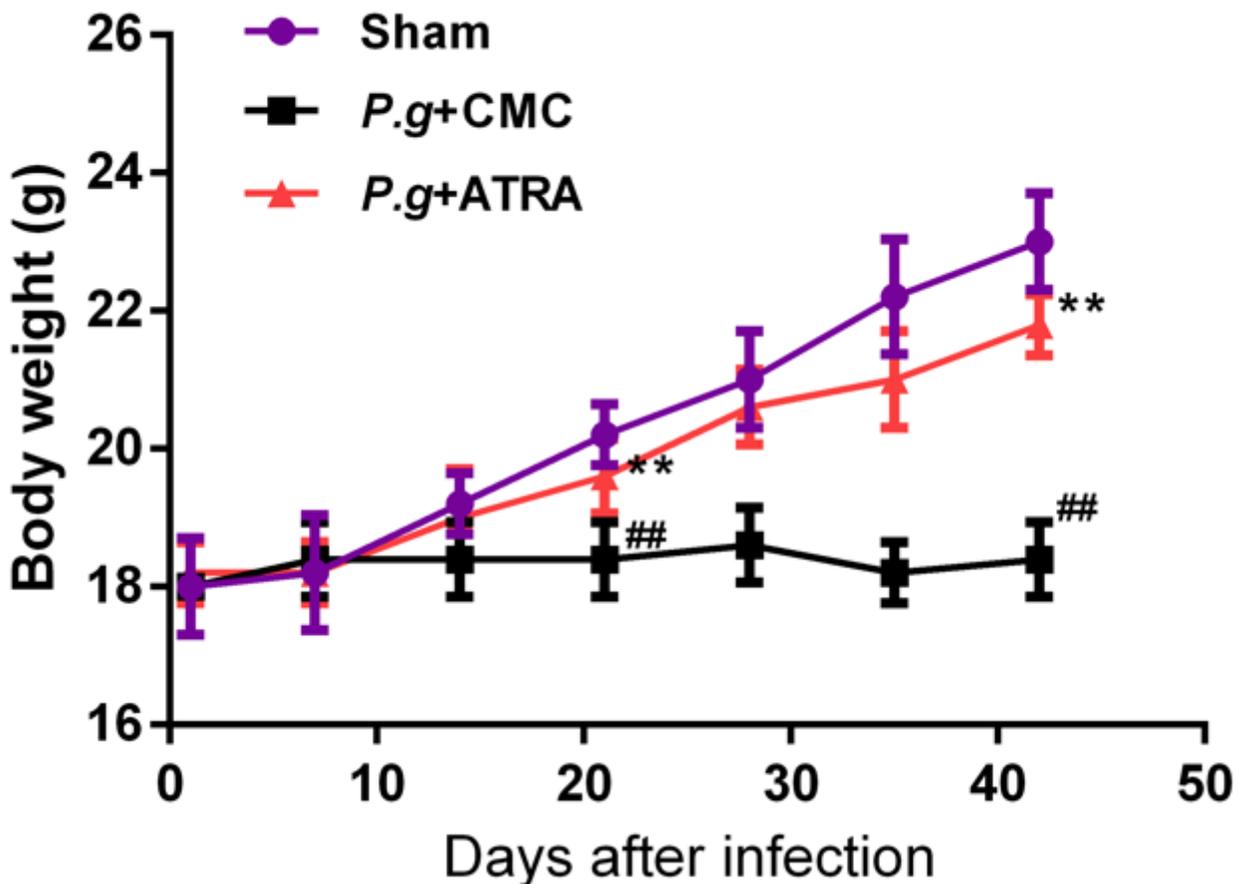


Figure 1

ATRA improved the body weight changes in periodontitis mice. Note: ## $P < 0.01$ vs. Sham group; ** $P < 0.01$ vs. P.g+CMC group.

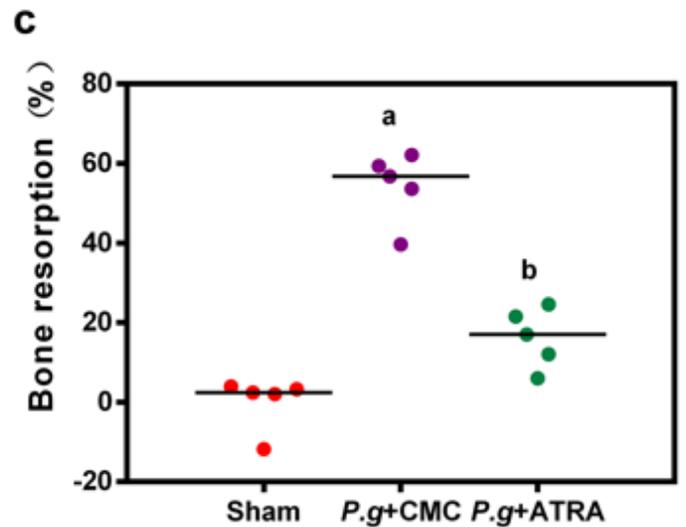
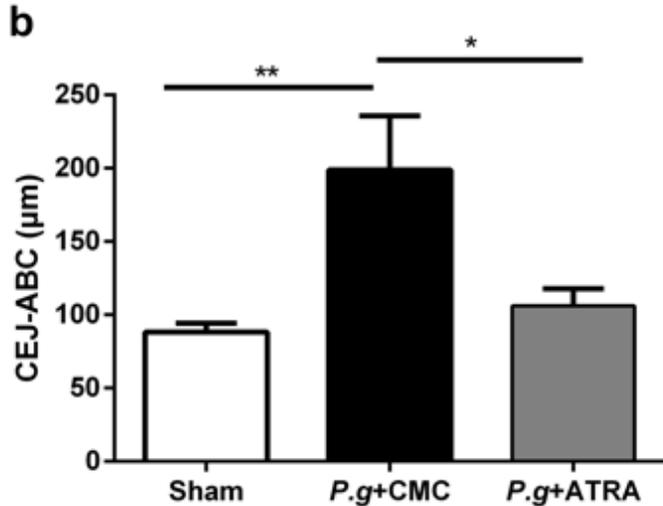
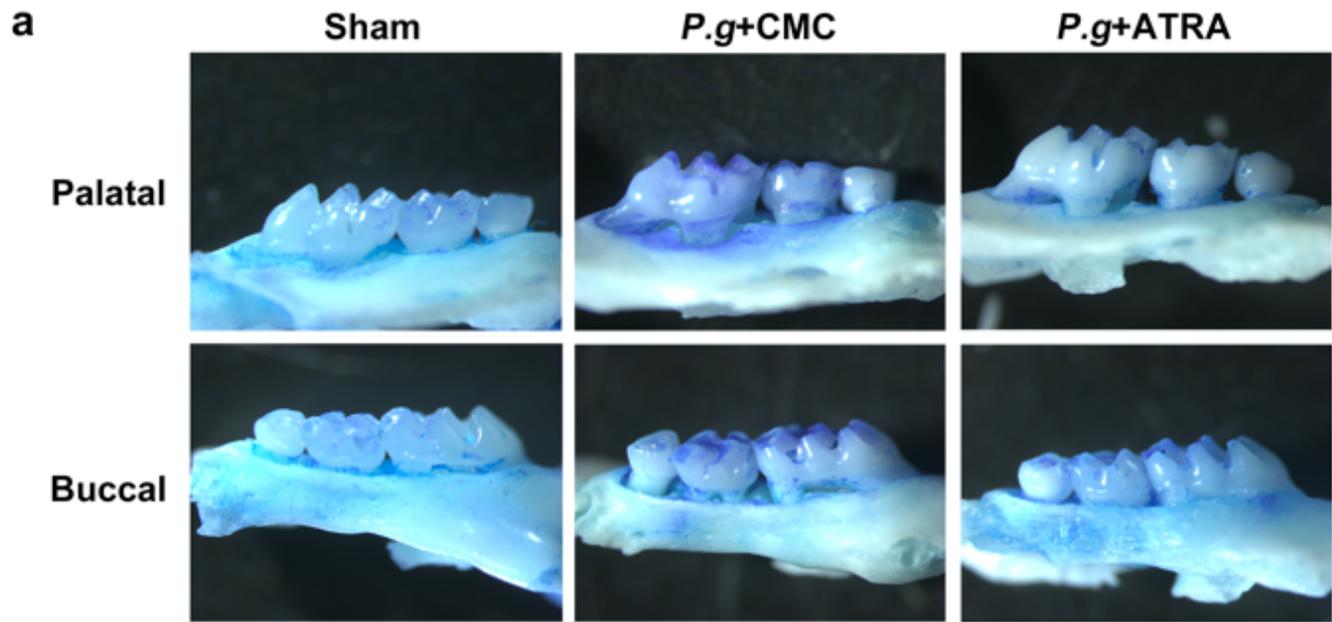


Figure 2

Evaluation of alveolar bone resorption in experimental periodontitis. (a, b) Alveolar bone resorption was analyzed by measuring the distance from the CEJ to the ABC on both the buccal and palatal molar surfaces. Representative images are shown (32 \times magnification) (a). Values are shown as the mean \pm SD of three mice per group. * $P < 0.05$, ** $P < 0.01$ (b). (c) Alveolar bone resorption rate. a: $P < 0.01$ vs. Sham group; b: $P < 0.01$ vs. *P.g*+CMC group.

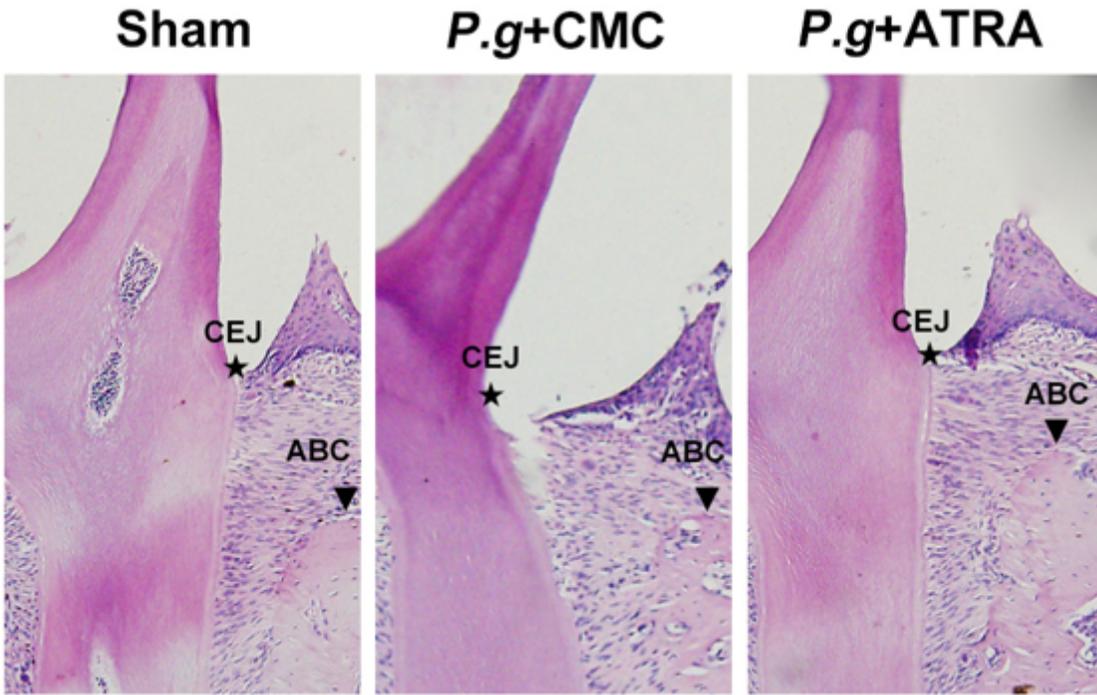


Figure 3

Histological examination of periodontal tissues (100× magnification). ABC, alveolar bone crest; CEJ; cemento-enamel junction.

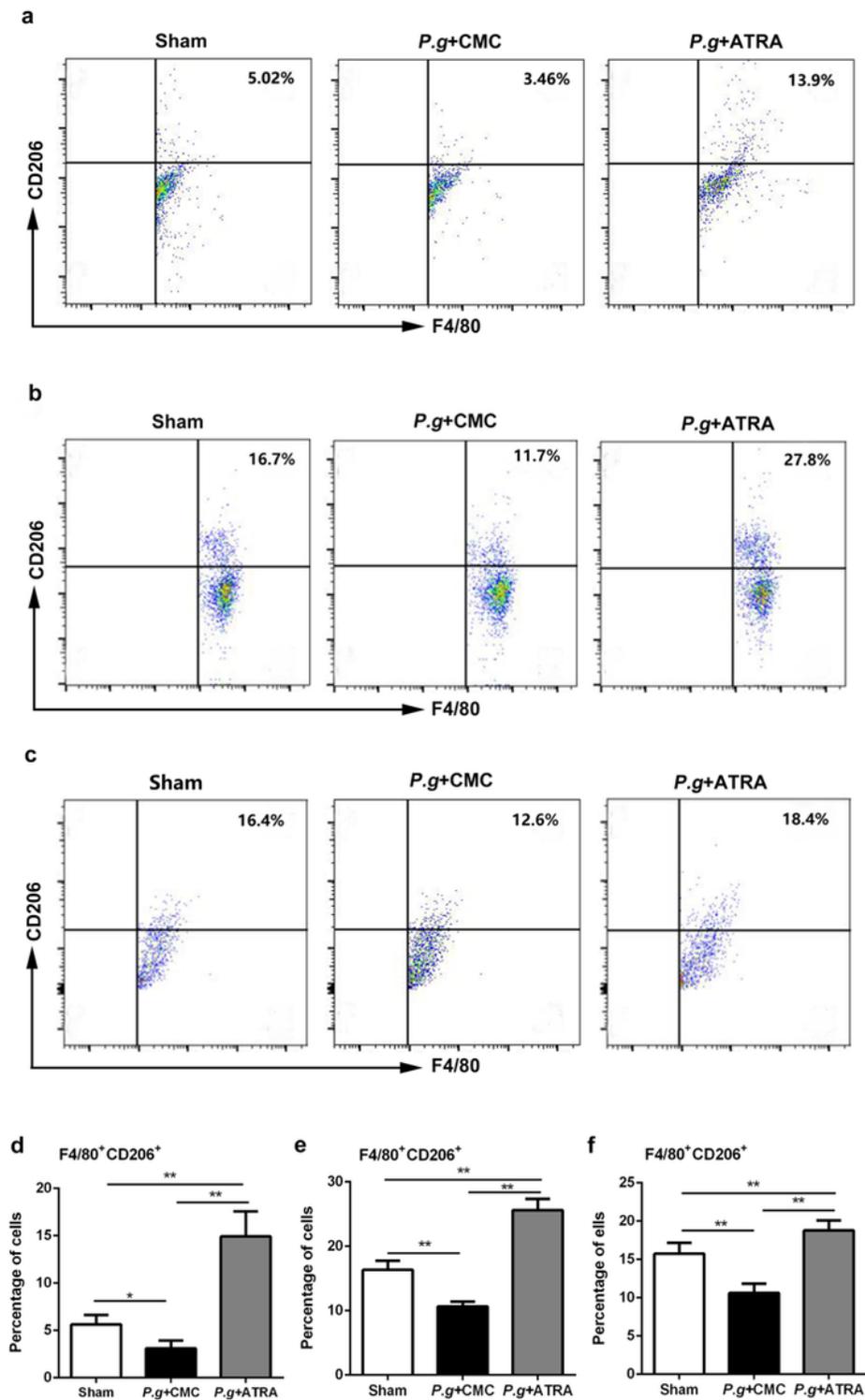


Figure 4

Oral administration of ATRA increased the proportion of M2 macrophages in *P. gingivalis*-infected mice. Flow cytometric analysis of M2 macrophages. Representative dot plots for F4/80⁺CD206⁺ (M2 macrophages) staining in (a) GMCs, (b) PLF, and (c) spleen. (d, e, f) Percentages of F4/80⁺CD206⁺ (M2 macrophages) in GMCs, PLF, and spleen, respectively. Data are shown as the means \pm SD of five mice per

group. *P < 0.05, **P < 0.01 between the two groups. GMCs, gingival mononuclear cells; PLF, peritoneal lavage fluid.

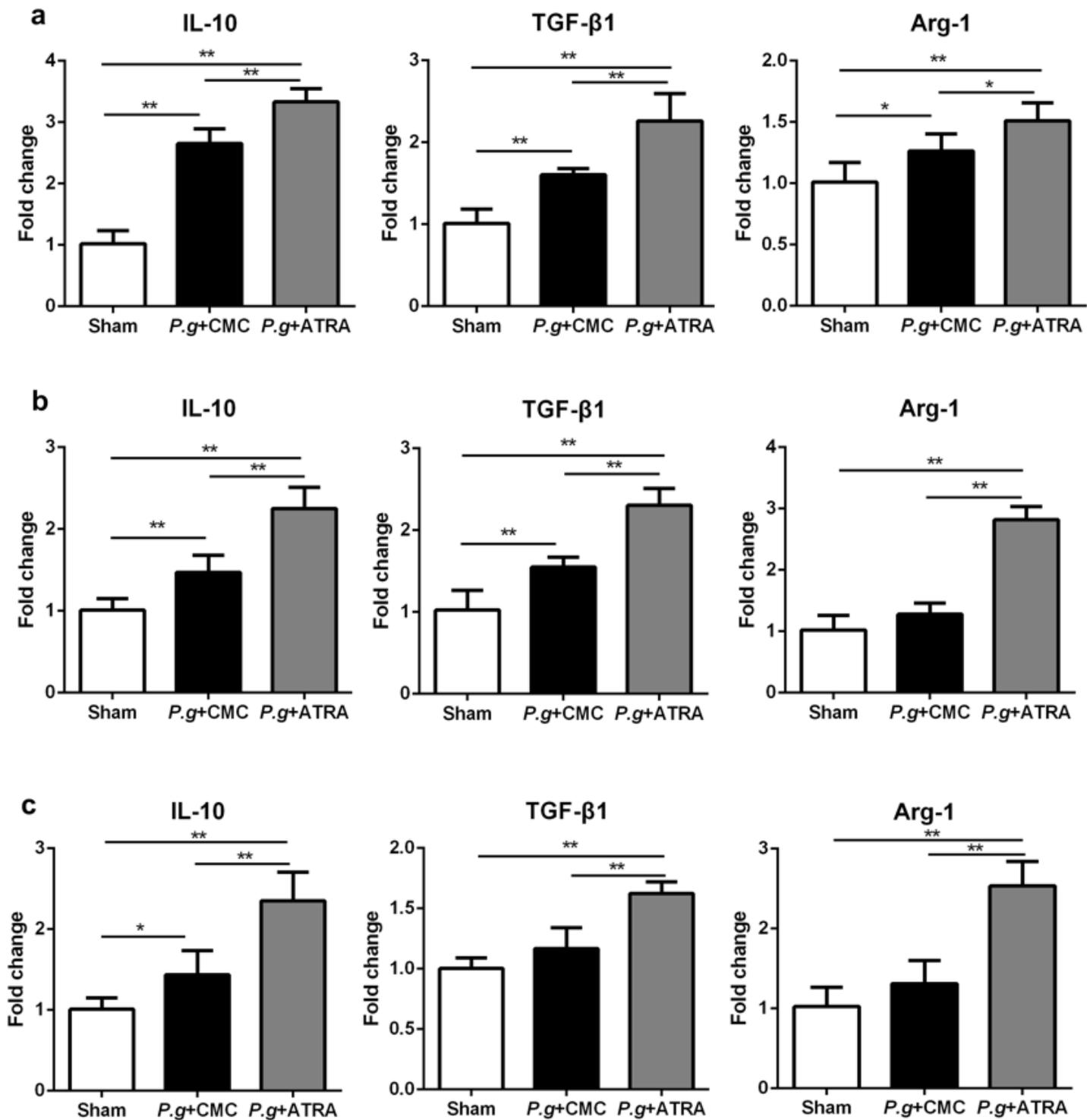


Figure 5

ATRA increased the mRNA expression of M2 macrophage-related cytokines in periodontitis. IL-10, TGF-β1, and Arg-1 mRNA expressions were determined by qRT-PCR and expressed as fold changes with

respect to levels in Sham mice (set as 1). (a–c) Fold changes (mean \pm SD) of IL-10, TGF- β 1, and Arg-1 mRNA in gingivae (A), PLF (b), and spleen (c). *P < 0.05, **P < 0.01.