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Purple red rice bran anthocyanins ameliorate inflammatory response induced by lipopolysaccharide from *Porphyromonas gingivalis* in RAW264.7 cells *via* suppressing NF-κB and AP-1 signaling pathways

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ABSTRACT

Periodontitis, a persistent inflammatory condition, targets the gums and periodontal structures, negatively impacting health. Systemic and local antimicrobials are often used to treat periodontitis, but they may lead to adverse effects such as drug resistance and ecological imbalance. As natural water-soluble pigment, anthocyanins have been reported to be an effective antiinflammatory, antiobesity, anticancer, antimicrobial and so on. The purpose of the study was to elucidate the anti-inflammatory effects and potential molecular mechanisms of purple red rice bran anthocyanins (PRBA) on RAW264.7 cells induced by lipopolysaccharide from *Porphyromonas gingivalis* (Pg-LPS). The results indicated that PRBA inhibited the expression of iNOS and COX-2 in RAW264.7 cells, reduced the level of NO, TNF-α, and IL-6, while increasing the expression of Arg-1 and the production of IL-10 in RAW264.7 cells. Among them, cyanidin-3-glucoside and peonidin-3-glucoside, the 2 main anthocyanin glycosides in PRBA, also had the same effects. RNA-seq results revealed that there were 1 840 differentially expressed genes (DEGs) after PRBA treatment, with 1 263 upregulated and 577 downregulated. Western blot also confirmed that PRBA significantly inhibited the activation of NF-κB and AP-1 in RAW264.7 cells. In summary, these findings suggested that PRBA inhibited the expression of proinflammatory cytokines in RAW264.7 cells induced by Pg-LPS through the NF-κB and AP-1 pathways, which providing its potential application in the treatment of periodontitis for anti-inflammatory effects.

1. Introduction

Periodontitis is the prevalent oral diseases and one of the most significant causes of tooth loss[1] . It can lead to inflammation of the gums and resorption of the alveolar bone, which in severe cases can cause loosening and loss of teeth[2]. *Porphyromonas gingivalis* (Pg) is the main pathogen of periodontitis[3]. Lipopolysaccharide generated from *Porphyromonas gingivalis* (Pg-LPS) is a major stimulus that evokes a host response in periodontal pockets and activates immune cells to release proinflammatory cytokines, thereby triggering the inflammation associated with periodontitis[4]. For example, Pg-LPS has the ability to stimulate macrophages to generate pro-inflammatory molecules like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)[5]. Macrophages are a crucial component of the innate immune system in the human body, playing a crucial role in host defense, maintaining internal environmental homeostasis, and inflammatory response[6]. When macrophages are activated by Pg-LPS, they manifest prominently in the inflamed periodontal region, inciting inflammation and worsening the periodontitis. Currently, the main treatment methods for periodontitis are teeth cleaning to remove plaque and tartar, as well as the use of antibiotics. But the use of antibiotics may result in resistance and other adverse effects. Therefore, the development of alternative ways to enhance periodontitis treatment is imperative.

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Anthocyanins are type of flavonoid found in many vegetables and fruits, giving them bright colors such as purple, blue, and red[7]. They are also one of the most widely distributed natural pigments in the plant community, and have a variety of biological properties, such as anti-inflammatory, antioxidant, antibacterial, and immunomodulatory activities[8-9]. The mechanism by which anthocyanins exert their anti-inflammatory effects has been reported to be the reduction of the inflammatory factors IL-6 and TNF-α through down-regulation of the gene expression level of nuclear factor kappa-B (NF-κB)[10]. The NF-κB signaling pathway is activated in response to inflammatory stimuli within cells, and it can induce the expression of various inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. The AP-1 signaling pathway involves the formation of dimers of c-Jun and c-Fos, which are transcription factors activated in response to inflammatory stimuli and cellular stress. Both the NF-κB and AP-1 signaling pathways are key regulatory routes in the inflammatory response. Purple red rice is a unique colored hybrid rice from Luxi County in Pingxiang City, Jiangxi Province, China, boasts a translucent purplish-red hue. It is highly nutritious, packed with protein, dietary fiber, vitamins, and trace elements. Notably, its rice bran is rich in anthocyanins, a type of flavonoid that serves as a natural water-soluble pigment.

Regulating inflammatory mediators could be a potential treatment for periodontitis. Anthocyanins have a variety of biological and pharmacological functions, although their potential anti-inflammatory effects on periodontitis are not thoroughly recognized. Taken together, this study aimed to study the anti-inflammatory effects of anthocyanins in macrophages RAW264.7 cells stimulated by Pg-LPS, and explore its underlying mechanisms utilizing RNA-Seq and Western blotting. The study is intended to offer a perspective and approach to treating periodontitis.

2. Materials and methods

2.1 Materials

Purple red rice bran was provided by Luxi Country (Jiangxi, China). RAW264.7 cells were kindly procured from the stem cell bank of the Chinese Academy of Science (Shanghai, China). Fetal bovine serum (FBS) was obtained from Vivacell Biosciences (Shanghai, China). Dulbecco's modified eagle medium (DMEM) was obtained from Solarbio (Beijing, China). TNF-α, IL-6, interleukin 10 (IL-10) ELISA kits, nitric oxide (NO) assay kits, nuclear/cytoplasmic protein extraction kit from Beyotime Biotechnology (Shanghai, China), primary antibodies cyclooxygenase 2 (COX-2), inducible nitric oxide sythase (iNOS) rabbit mAb were purchased from Cell Signaling Technology (Massachusetts, USA). Arg-1, p-jun, c-fos, LaminB 1, NF-κB p65, IkBα, and p-IkBα were sourced from proteintech (Wuhan, China). The related secondary antibodies were obtained from Zhong Shan Golden Bridge Biological Technology Co. Ltd. (Beijing, China). Cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) were procured from Caoyuankang Biotechnology Co., Ltd. (Chengdu, China).

2.2 Preparation of purple red rice bran anthocyanins (PRBA)

According to the related literature[11], 50% acidic ethanol method was used for the extraction of PRBA. An appropriate amount of dried purple red rice bran powder was weighed and pH 2, 50% ethanol was added at a ratio of 1:10 (g/mL). The rice bran was extracted at 50 °C in a water bath for 60 min. The supernatant was then collected by centrifugation at 4 800 r/min for 10 min. After repeating the extraction twice, all the filtrates were combined, and petroleum ether was added to remove the oils. The filtrate was concentrated in vacuum, freeze-dried to obtain the PRBA, dried and kept in a dry place away from light.

2.3 Cell culture

The RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a 5% CO_2 cell culture incubator. RAW264.7 cells were cultured in 96-well plates for 24 h, then supernatant was removed from the cells. Add equal volumes of PRBA (0–1 000 μg/mL) or Pg-LPS (1 μg/mL) to the wells and use for analysis after stimulating the cells for 24 h under the same conditions.

2.4 Cell viability assay

The proliferative effect of PRBA treatment on RAW264.7 cells was determined by CCK-8 as descrbed in Qin et al.[12]. After overnight adherent culture of RAW264.7 cells, the medium in well plates was aspirated, the cells were treated with different concentrations of PRBA (50, 100, 150 and 200 μg/mL) for 24 h. The blank group was a cell-free CCK-8 mixture. After 24 h of incubation, the supernatant was discarded and washed twice with PBS, then 100 μL 10% CCK-8 was added and incubated for 30 min. The absorbance was measured at 450 nm using the Varioskan Flash System Enzyme Labeler (Thermo Scientific, USA). The formula was calculated as follows:

$$\text{Proliferation rate (\%)} = \frac{OD_{PRBA} - OD_{Blank}}{OD_{Control} - OD_{Blank}} \times 100$$

2.5 NO and ELISA assay

Culture supernatants were gathered and levels of NO and TNF-α, IL-6 and IL-10 were measured using commercial Griess kits or ELISA kits following the instructions of the manufacturer.

2.6 Western blotting analysis

RAW264.7 cells were collected after being treated with PRBA for 24 h. Cell protein concentration was measured using BCA protein kit. The membrane was blocking with 5% BSA solution and incubated overnight at 4 °C with primary antibody. Then the membrane was washed 3 times with TBST and exposed for 50 min with a suitable secondary antibody. Washed 3 more times, proteins were measured with an ECL kit and results were visualized with a ChemiDoc™ XRS imaging system. Quantification of the bands was performed using Image Lab software.

2.7 Anti-inflammatory assay of PRBA monomers

The anthocyanin monomers C3G and P3G were selected for cellular experiments. Monomer concentrations of 1, 2, 4, 10, 20, 50, and 100 μmol/L were selected for cell viability assays, and drug

concentrations with no apparent effect on cell viability were used for ELISA and Western blotting tests. The specific procedure was the same as section 2.5 and 2.6.

2.8 RNA-seq analysis

RAW264.7 cells with a concentration of 2×10^5 cells/mL were cultured for 24 h. Then removed the supernatant and PRBA at 200 μg/mL was added into the incubator for further culture. After that, the cells were collected, total RNA was extracted and purified from the cell samples using the Trizol kit as directed by the manufacturer. The preparation of the library and sequencing were performed at Novogene Co., Ltd. (Beijing, China).

2.9 Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA extracted as described in section 2.8 was back-transcribed into cDNA with a PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Dalian, China), following the instructions of the manufacturer. The resulting cDNA was subsequently utilized for qRT-PCR analysis with the TB Green® Premix Ex *Taq*™ kit (Takara Bio, Dalian, China) on a CFX Connect PCR system (Bio-Rad Laboratories Co., Ltd., USA). Relative expression levels of the target genes were determined by the $2^{-\Delta\Delta CT}$ method, with GAPDH serving as the internal control. Each sample included 3 biological replicates, and the entire experiment was conducted in triplicate. Primer sequences for the target genes were provided in Table 1.

Table 1
Primer sequences of RT-qPCR.

Gene	Forward primer sequences (5′-3′)	Reverse primer sequences (3′-5′)
Lcn2	TCTGTCCCCACCGACCAAT	GGAAAGATGGAGTGGCAGACA
Irf7	GGGACCTCTTGCTTCAGGTT	CAAGGCTGCGCTCAGGA
Cxcl2	CCAAAAGATACTGAACAAAGGCA	CGAGGCACATCAGGTACGA
Atf3	AGCGAAGACTGGAGCAAAATG	ATACCAGTGACCCAGGAGGT
Ccl4	CTGTGCAAACCTAACCCCGA	AGGGTCAGAGCCCATTGGT
Ccl5	GTGCCCACGTCAAGGAGTAT	TCGAGTGACAAACACGACTG
Ccl3	CCAAGTCTTCTCAGCGCCATA	TCTCTTAGTCAGGAAAATGACACC
Jund	GGCGGGATTGAAACCAGGG	AGCCCGTTGGACTGGATGA
Ccl2	CACTCACCTGCTGCTACTCA	GCTTGGTGACAAAAACTACAGC
Cxcl10	CCACGTGTTGAGATCATTGCC	TCACTCCAGTTAAGGAGCCC
Tgfbr1	CCACAAACAGTGGCGGC	AAACACTGTAATGCCTTCGCC
Cd40	TTGTTGACAGCGGTCCATCT	TCTCAAGAGCTGTGCAGTGG
Atf5	TATGAAGAGGAATAAGATGAGGTCC	CTGCACCAACAATTGGGAGG
H2-T23	AGAGTAACGACGAATCTCACACG	CTTGCAGGTATGCCCTCTGTT
GAPDH	CAGGAGAGTGTTTCCTCGTCC	*TGAGGTCAATGAAGGGGTCG*

2.10 Statistical analysis

The data were performed by applying SPSS 26.0 (IBM software, NY, USA), and data for quantitative studies were presented as mean ± standard deviation. Data were analyzed by One-way ANOVA or independent samples *t*-test. $P < 0.05$ was considered statistically significant. GraphPad Prim 9.0.2 (GraphPad Software Inc., San Diego, USA) was used for statistical plotting of the experimental data.

3. Results

3.1 Cell viability

The cytotoxicity of anthocyanins on RAW264.7 cell viability was evaluated by CCK-8 assay and the results are demonstrated in Fig. 1A. PRBA showed no significant effect on the activity of RAW264.7 cells in the concentration ranging from 50 to 200 μg/mL. However, when the concentration reached 400 μg/mL, PRBA began to inhibit the RAW264.7 cells. And the cell viability was reduced progressively with increasing anthocyanins concentration. Research also showed that as the concentration of anthocyanins increases, anthocyanins have a certain inhibitory effect on cell growth.

3.2 Effects of PRBA on inflammatory cytokine production

NO is a liposoluble gas molecule with free radical properties, it can participate in or affect various biochemical reactions in the body[13], thus it is an essential factor in regulating the activity of osteoclasts, and also considered a marker for macrophage activation[14]. Activated macrophages are capable of secreting a series of cytokines, stimulating cellular or humoral immune responses[15]. The Griess method and ELISA were used to determine the effect of PRBA on the secretion of NO and cytokines (TNF-α, IL-6, and IL-10) by RAW264.7 cells. Fig. 1B shows the changes in NO secretion levels induced by PRBA in RAW264.7 cells, indicating that different concentrations of PRBA can inhibit the NO secretion ability of RAW264.7 cells. A significant difference ($P < 0.01$) was observed in NO secretion levels between the PRBA and Pg-LPS groups. Simultaneously, a similar effect of PRBA on the secretion of TNF-α and IL-6 by RAW264.7 cells was observed (Figs. 1C and D). PRBA inhibited the growth in TNF-α and IL-6 concentrations than that of the Pg-LPS group. Moreover, the cytokine levels decreased dosedependently with increasing PRBA concentration. Notably, PRBA had the lowest TNF-α and IL-6 concentrations of 200 μg/mL. PRBA promoted the secretion level of the anti-inflammatory factor IL-10, which increased with increasing concentration (Fig. 1E). These results suggested that PRBA exerted anti-inflammatory effects by inhibiting the production of NO and cytokines by RAW264.7 cells.

iNOS is the key enzyme for nitric oxide synthesis[16]. The activation of iNOS catalyzes oxidative deamination, which has detrimental effects on the pathogenic cascade of inflammatory diseases. Furthermore, COX-2 is an inflammatory enzyme that can lead to endothelial dysfunction in periodontitis[17]. Both iNOS and COX-2 are considered proinflammatory enzymes, and COX-2 is also regarded as a target in inflammatory therapies[18]. Arg-1 is a marker of M2 macrophage polarization, representing an anti-inflammatory factor[19]. To further elucidate the possible mechanisms of PRBA on Pg-LPS-induced anti-inflammatory activity in macrophages, we performed protein level analysis to determine the effects of PRBA on iNOS, COX-2 and Arg-1 protein expression. As shown in Figs. 2A-D, PRBA induced a concentration-dependent decrease in iNOS protein expression in range of 50 to 200 μg/mL. The expression of iNOS and COX-2 markedly increased in the Pg-LPS group, while the expression of Arg-1 significantly decreased when compared with the untreated group. However, treatment of cells with PRBA resulted in a reduction in the expression of iNOS and COX-2 and an increase in the expression of Arg-1.

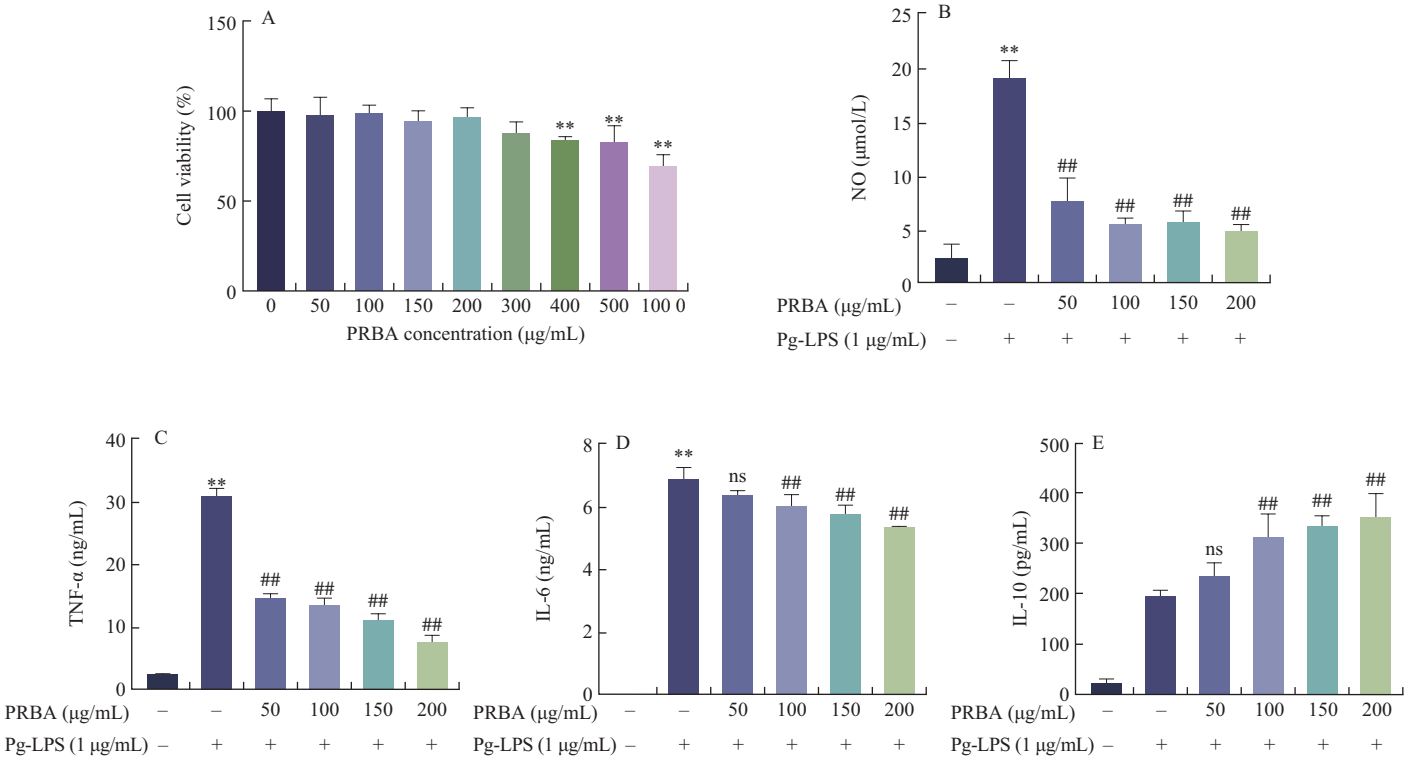

Fig. 1 Effect of different concentrations of PRBA on the viability, as well as NO and cytokine secretion of RAW264.7 cells. (A) Cell viability, (B) NO, (C) TNF-α, (D) IL-6, (E) IL-10. Differences as compared with blank group (*$P < 0.05$, **$P < 0.01$), and model group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). ns. not significant.

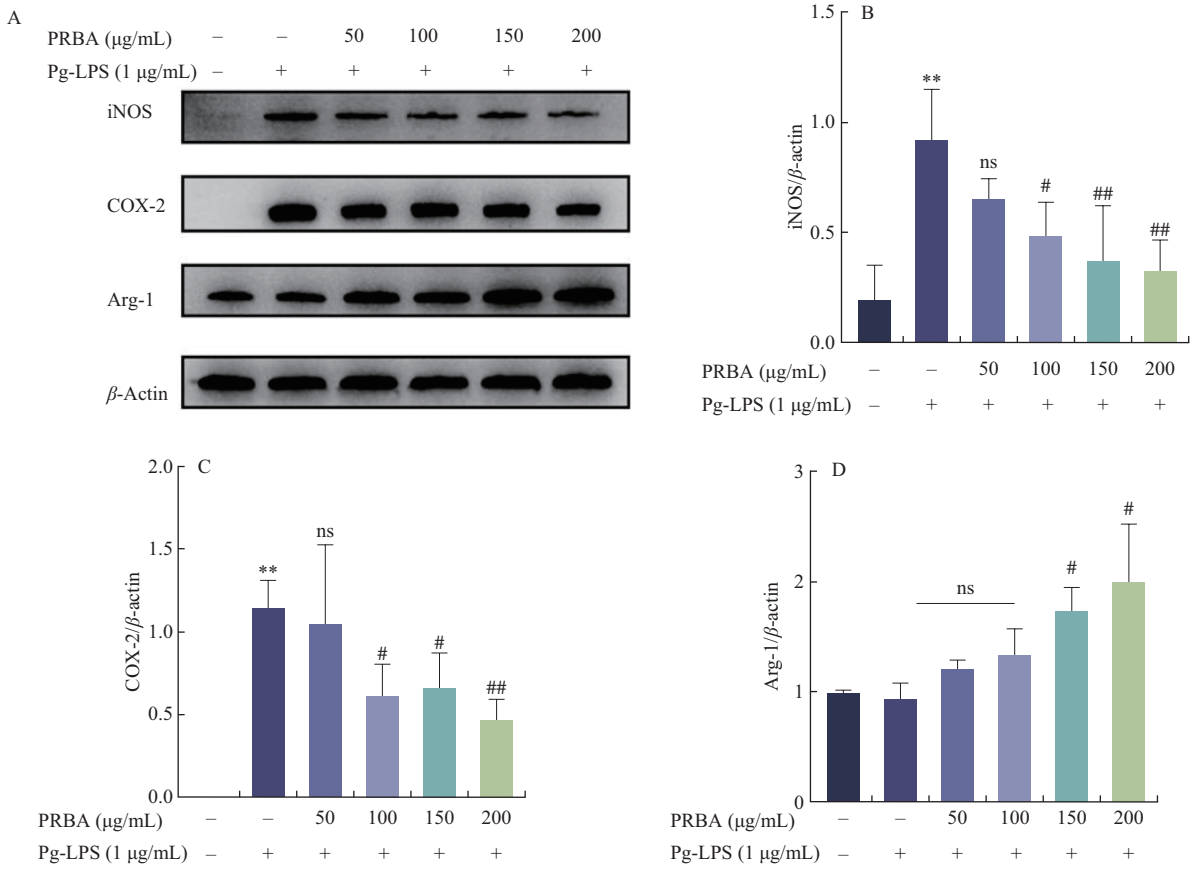

Fig. 2 PRBA inhibited the activation of iNOS, COX-2, and Arg-1 in macrophages. (A) Western blotting analysis of proteins; (B-D) Bar graphs represent the quantitative expression levels of iNOS, COX-2, and Arg-1, respectively. Differences as compared with blank group (*$P < 0.05$, **$P < 0.01$), and model group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). ns: not significant.

3.3 Effect of anthocyanin monomers on cell viability

C3G and P3G, the 2 major anthocyanins in PRBA, were selected for the experiment. To evaluate their anti-inflammatory activity, the toxicity of RAW264.7 cells was measured. The results indicated that within the concentration range of 1–20 μmol/L, P3G and C3G showed no cytotoxicity to RAW264.7 cells (Figs. 3A and B). Therefore, P3G and C3G at concentrations of 2, 4, 10, and 20 μmol/L were chosen for further studies.

3.4 Effect of anthocyanin monomers on cytokine production

The effects of P3G and C3G on TNF-α and IL-6 were mainly determined here. After treatment with P3G and C3G, the contents of TNF-α and IL-6 in RAW264.7 cells were considerably reduced ($P < 0.01$) (Figs. 3C-F). Furthermore, as the concentration of the treatment increased, the content of inflammatory factors decreased in a dose-dependent manner.

3.5 Effect of anthocyanin monomers on COX-2 protein expression

As depicted in Figs. 4A-D, with the increase in the concentrations of P3G and C3G, the protein level of COX-2 dramatically declined at a dose-dependent rate. This indicated that both P3G and C3G could inhibit the expression of the inflammatory protein COX-2. Overall, C3G exhibited a better inhibitory effect.

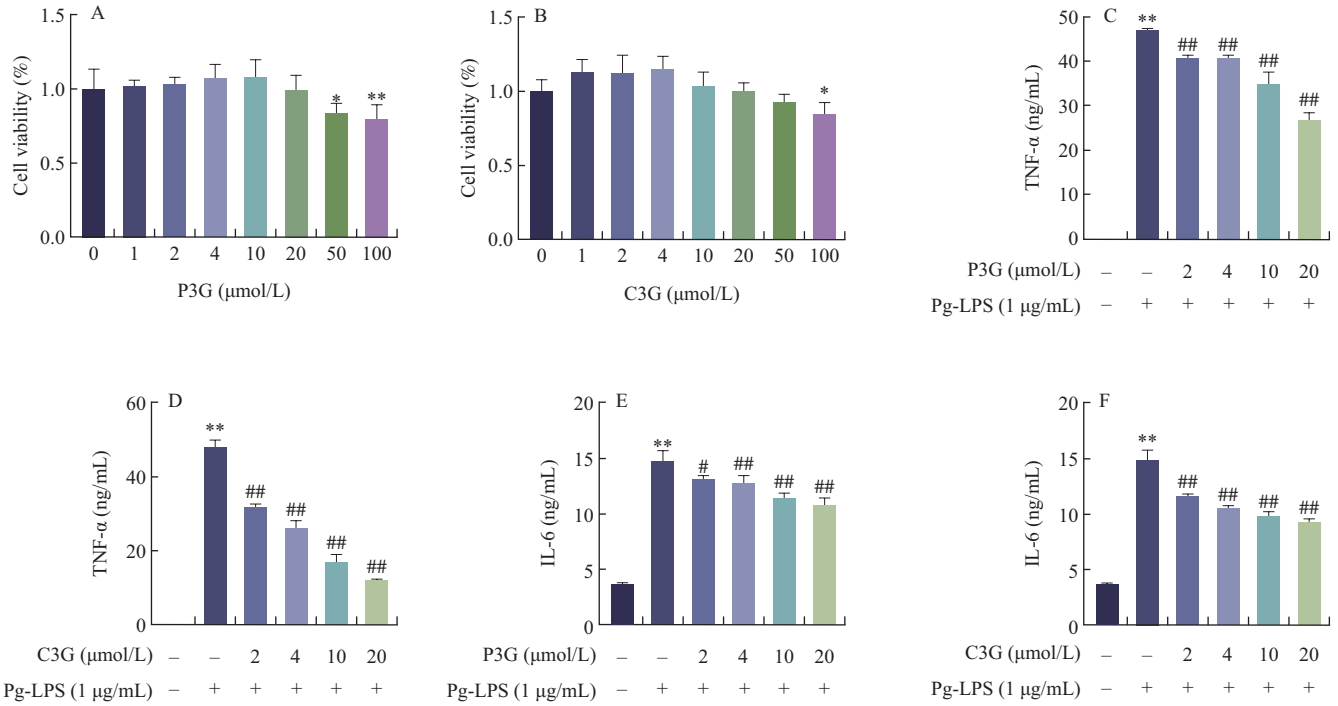

Fig. 3 Effect of P3G and C3G on viability and cytokine secretion by RAW264.7 cells. (A, B) Different concentrations of P3G and C3G on the viability of RAW264.7 cells; (C, D) TNF-α; (E, F) IL-6. Differences as compared with blank group (*$P < 0.05$, **$P < 0.01$), and model group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

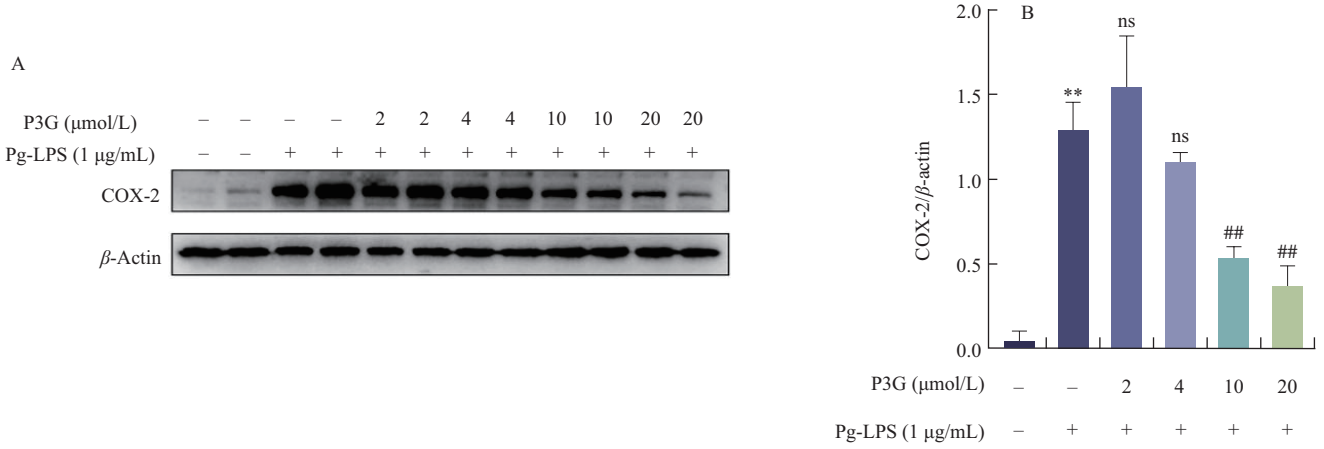

Fig. 4 P3G and C3G inhibited the activation of COX-2 in macrophages. (A, C) Western blotting analysis of protein; (B, D) Bar graphs represent the quantitative expression levels of COX-2. Differences as compared with blank group (*$P < 0.05$, **$P < 0.01$), and model group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). ns: not significant.

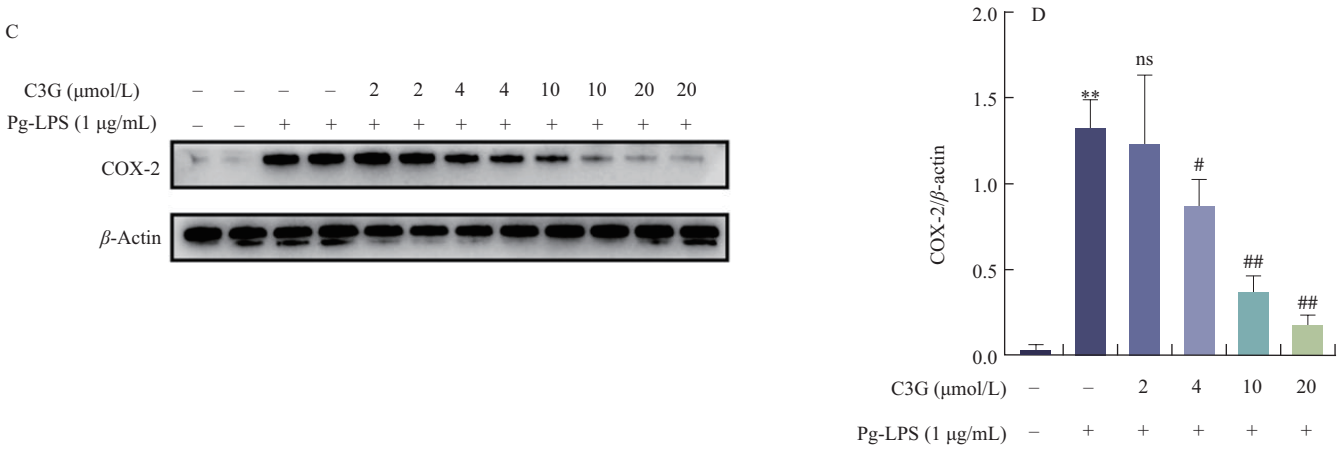

Fig. 4 (*Continued*)

3.6 *Differentially expressed mRNAs*

In order to further understand the anti-inflammatory mechanism of PRBA on RAW264.7 cells, transcriptome analysis was conducted using RNA-seq technology. Correlation analysis revealed that there was good intra-group parallelism and significant inter-group differences among the samples (Fig. 5A). PCA analysis was carried out on the nine samples according to their expression levels, and the results are presented in Fig. 5B. Each group of samples was represented by a different color, and there was a clear distinction between the three-color markers, demonstrating significant differences between the NC, PgLPS, and PRBA group. Meanwhile, the proximity of samples with the same color marker indicated a high similarity among them. The differential expression analysis revealed significant differences in 332 genes between the NC group and the Pg-LPS group ($P_{adj} < 0.05$, $|\log_2 \text{fold change}| > 1$), with 279 upregulated genes and 53 downregulated genes (Fig. 5C). Meanwhile, there were 1 840 genes with significant differential expression between the PRBA group and the Pg-LPS group ($P_{adj} < 0.05$, $|\log_2 \text{fold change}| > 1$), including 1 263 upregulated genes and 577 downregulated genes (Fig. 5D), indicating that PRBA treatment could extensively regulate gene expression. The vertical axis represented variously expressed genes, where dark red suggested genes with high expression and green indicated low-expression genes (Fig. 5E). These results suggested that PRBA had reprogrammed genes expression in RAW264.7 cells.

3.7 *RT-qPCR to validate sequencing results*

To verify the reproducibility of the RNA-seq sequencing results, 13 differentially expressed genes (DEGs) (Fig. 5F) in the NC, Pg-LPS and PRBA groups were randomly selected for RT-qPCR experiments, with *GAPDH* as an internal reference. PRBA treatment markedly promoted the expression of *Tgfbr1* ($P < 0.01$) and significantly suppressed the expression of *Lcn2*, *Irf7*, *Atf3*, *Ccl4*, *Ccl5*, *Ccl3*, *Jund*, *Ccl2*, *Cxcl10*, *Cd40*, and *H2-T3* ($P < 0.01$) (Fig. 5G).

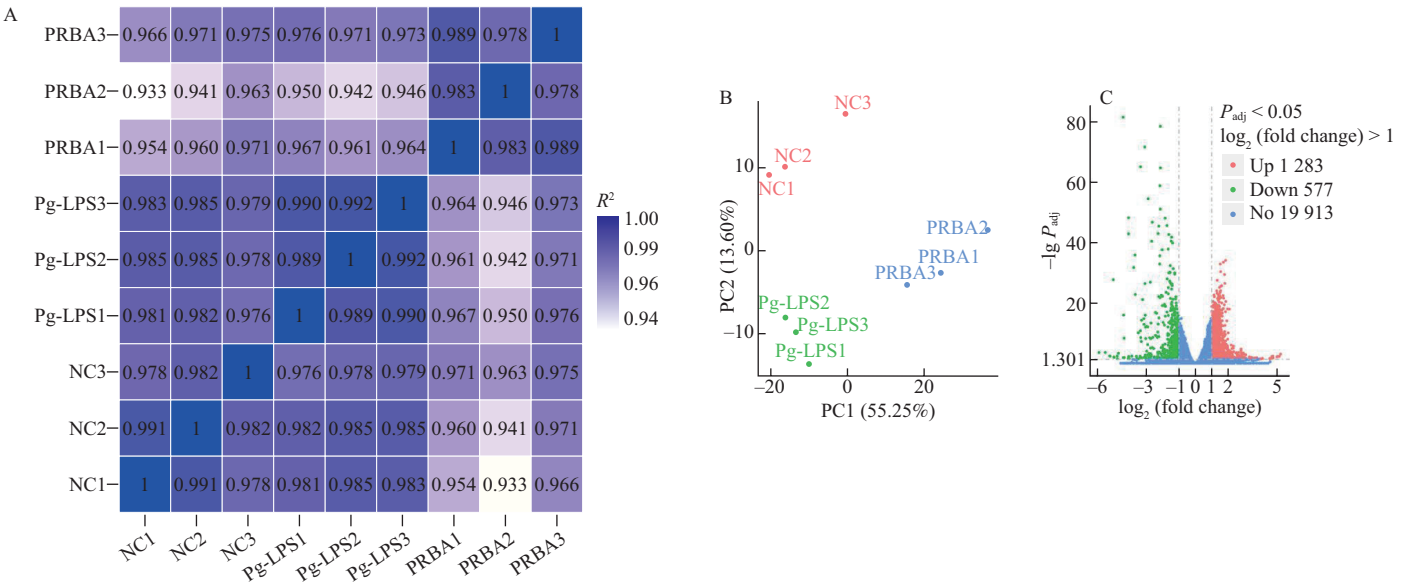

Fig. 5 PRBA-induced changes in mRNA expression profiles in RAW264.7 cells. (A) Sample correlation validation; (B) PCA analysis; (C) Volcano plot of DEGs between the NC and Pg-LPS groups; (D) Volcano plot of DEGs between the PRBA-treated and Pg-LPS groups; (E) Heat map representation of DEG between different groups; (F) Heatmap of relative expression of random genes in the RNA-seq section; (G) mRNA levels of 13 DEGs were detected by RT-qPCR.

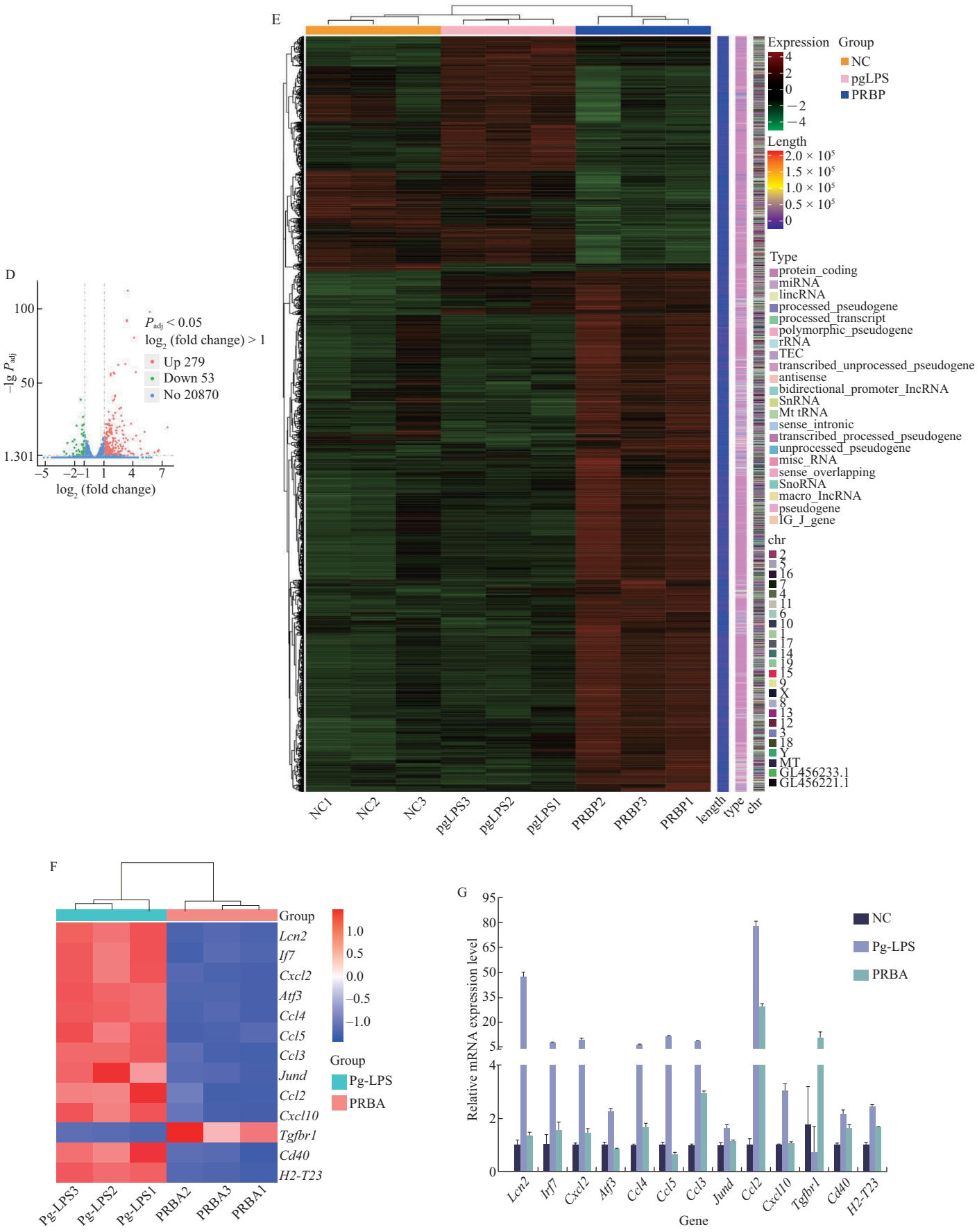

Fig. 5 (*Continued*)

3.8 Bioinformatics analysis

To study the biological functions of DEGs under PRBA treatment compared to Pg-LPS, we obtained relevant functional set information from the GO database, which interprets the significance of functional sets and identifies altered genes. The main enrichment pathways for GO functional enrichment analysis include biological process (BP), cellular component (CC), and molecular function (MF). The terms with the highest degree of enrichment from these 3 major categories are displayed in Figs. 6A and B, with the *x*-axis representing the most notably enriched GO terms, and the *y*-axis indicating the number of genes enriched in a specific GO term to the number of up- or down-regulated DEGs. Among the DEGs, the most prominent and abundant terms in BP were "response to viruses", "regulatory defense response" and "defense response to virus". In CC, the most significant and prevalent terms were "condensed chromosome", "centrosome", and "chromosome region". As for MF, the most outstanding and abundant terms were "cytokine receptor binding", "kinase regulator activity", and "double-stranded RNA binding". It is suggested by these results that PRBA may influence DEGs to exhibit anti-inflammatory effects. Based on the changes in DEGs, we analyzed the potential signaling pathways of PRBA-induced RAW264.7 macrophages. The extent of KEGG enrichment was assessed by enrichment factor, *P*-value, and the number of genes enriched in that pathway. Among them, a higher enrichment factor indicated a higher degree of enrichment, and a lower *P*-value indicated a more remarkable degree of enrichment. Figs. 6C and D illustrate the KEGG enrichment of the top 20 canonical pathways, including environmental information processing, cellular processes, organismal systems, human diseases, and metabolism. Among them, "Herpes simplex virus 1 infection", "NF-κB signaling pathway", and "TNF signaling pathway" were associated with inflammatory diseases in the body. Among them, the NF-κB and AP-1 signaling pathways were especially notable, suggesting that PRBA could use these pathways to mediate its anti-inflammatory effects.

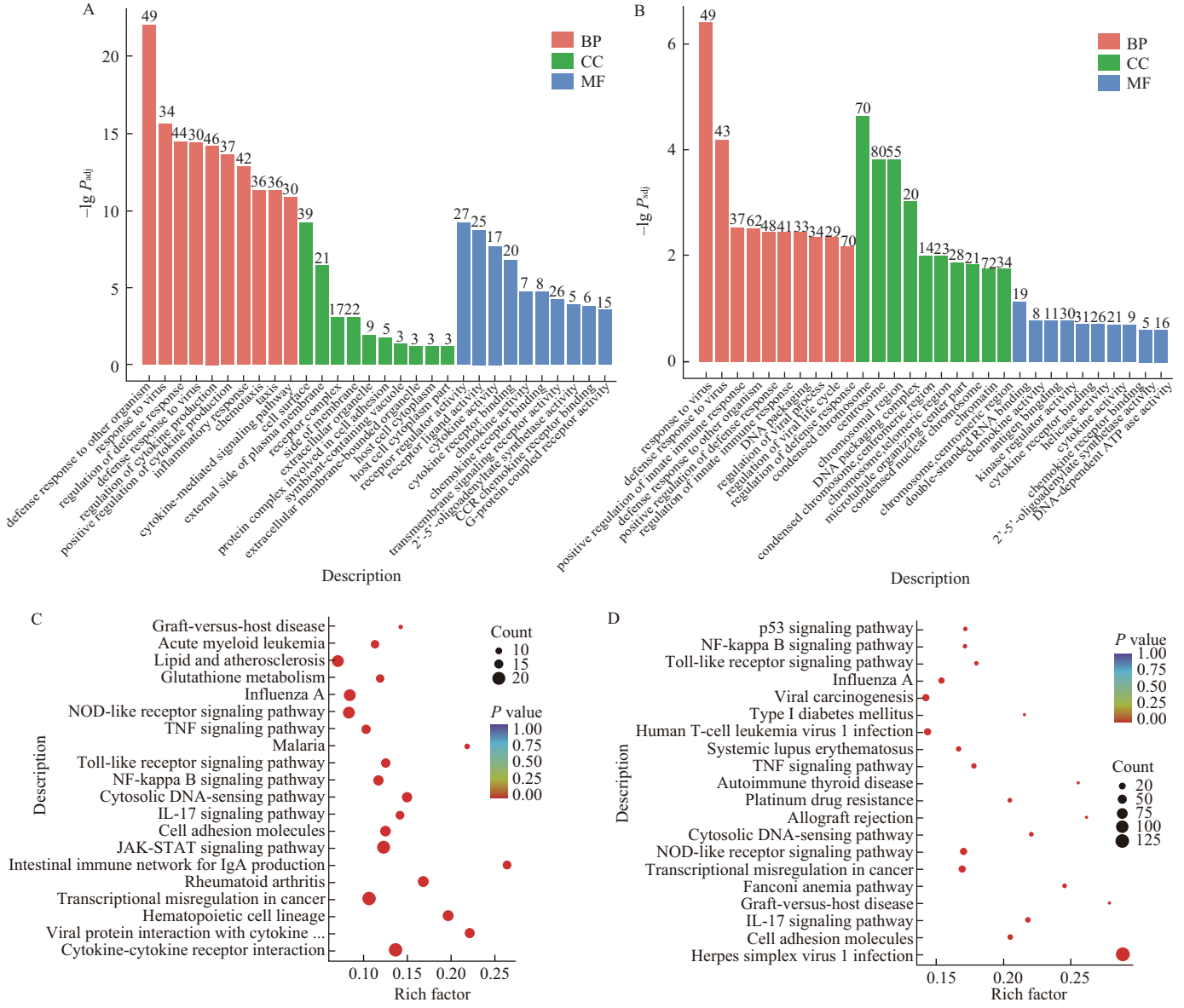

Fig. 6 GO and KEGG pathway enrichment analysis of DEGs. GO functional enrichment analysis of DEG between (A) NC and Pg-LPS groups, and (B) PRBA and Pg-LPS groups (B). KEGG pathway enrichment analysis of DEGs, (C)Pg-LPS *vs*. NC, (D) Pg-LPS *vs.* PRBA.

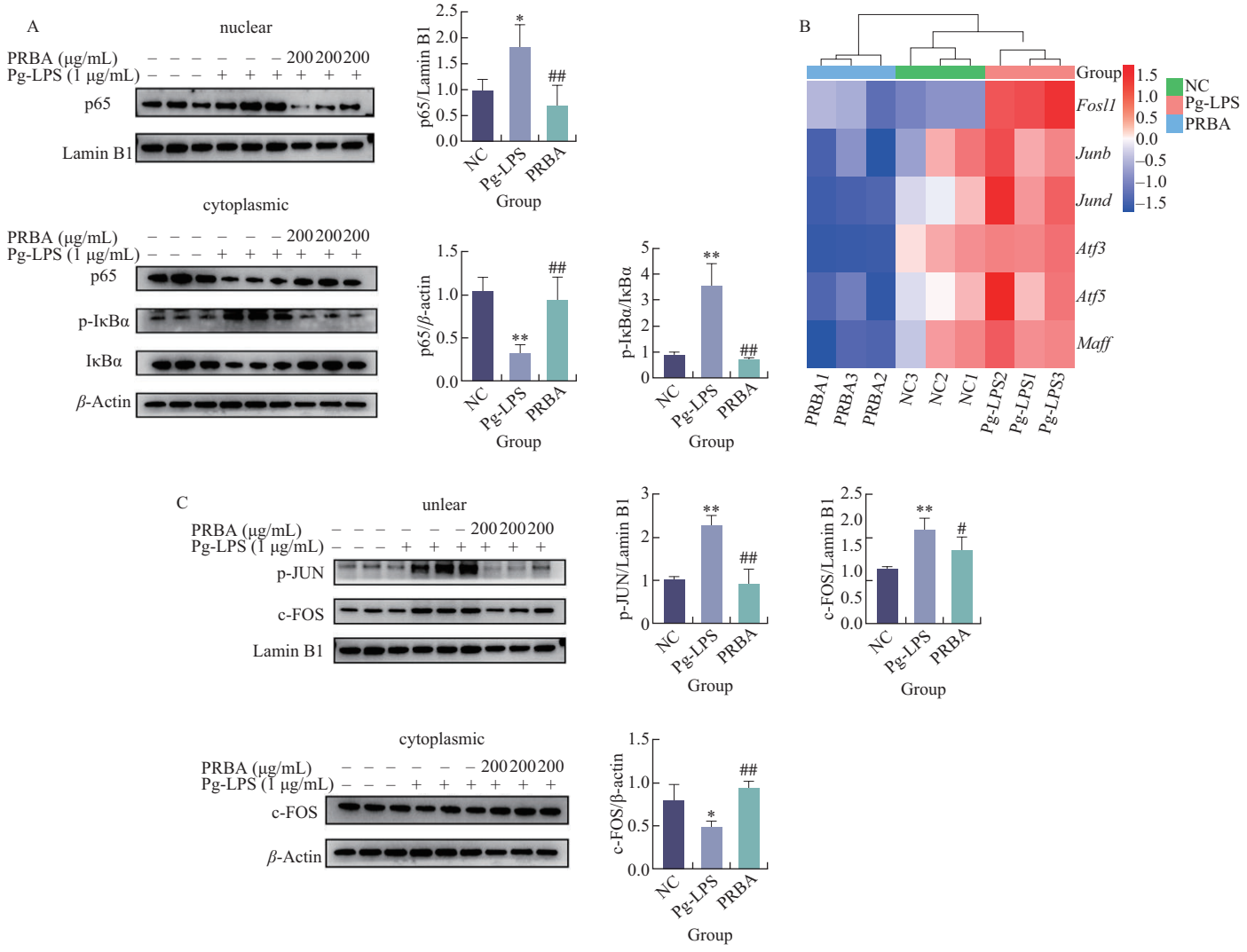

Fig. 7 Validating the signaling pathways involved in PRBA-induced RAW264.7 cell activation. (A) Representative bands and relative intensities of the NF-κB signaling pathway in the cytoplasm and nucleus; (B) Heatmap showing the major up- and down-regulated DEGs in the AP-1 pathway; (C) Representative bands and relative intensities of the AP-1 signaling pathway in the cytoplasm and nuclear. Differences as compared with blank group (*$P < 0.05$, **$P < 0.01$), and model group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). ns: not significant.

3.9 Validation of NF-κB and AP-1 pathway involvement in PRBA-activated RAW264.7 cells

To further validate the KEGG analysis, western blot analysis was conducted to examine the expression of important proteins in the NF-κB and AP-1 pathways. IκBα and p65 in the NF-κB signaling pathway generally existed in the cytoplasm in an inactive (non-phosphorylated) form. When cells were stimulated, IκBα become phosphorylated, triggering the activation of p65 and translocation to the nucleus[20]. AP-1, a heterodimer, made up of 2 subunits, c-JUN and c-FOS, and it serves as a primary component in regulating inflammatory responses. Activation of AP-1 was indicated when the c-FOS, and c-JUN families dimerized and moved from the cytoplasmic compartment into the nucleus[21]. Therefore, cytoplasmic and nuclear proteins of RAW264.7 cells were isolated independently. The results of PRBA treatment indicated that p65 expression in the nucleus was dramatically reduced ($P < 0.01$), although p65/β-actin levels in the cytoplasm were significantly elevated (Fig. 7A). The AP-1-related genes and expressions in the RNA-seq results are shown in Fig. 7B, and the c-FOS and p-JUN proteins were selected for subsequent validation. The ratios of c-FOS and p-JUN within the nucleus to the nuclear reference Lamin B1 were significantly reduced (Fig. 7C). Additionally, there was a significant rise in the cytoplasmic level of c-FOS ($P < 0.01$). These results indicate that the NF-κB and AP-1 pathways in RAW264.7 cells were significantly inhibited after PRBA treatment, attenuating the inflammatory response of the body.

4. Discussion

Anthocyanins have garnered widespread attention due to their remarkable anti-inflammatory and antioxidant properties. Previous research has validated the favorable anti-inflammatory properties of anthocyanins derived from fruits, vegetables, and rice[22-24], however, there is relatively limited research on the anti-inflammatory activity of PRBA. The objective of this investigation was aimed to examine whether PRBA could inhibit the inflammatory response that Pg-LPS elicited in RAW264.7 cells and explore its anti-inflammatory mechanism, which is associated with the development of periodontal disease.

The primary pathogen of periodontal disease, Pg, causes an inflammatory response in the gingiva[25]. Upon stimulation by Pg-LPS, macrophages release inflammatory mediators, which play a crucial role in the advancement of periodontitis. To investigate the anti-inflammatory effects of PRBA, we examined its impact on the production of inflammatory mediators induced by Pg-LPS. The findings demonstrated that PRBA significantly inhibited the secretion of inflammatory cytokines, including NO, TNF-α, and IL-6, induced by Pg-LPS in RAW264.7 cells, while promoting the generation of the anti-inflammatory factor IL-10. The viability of RAW264.7 cells was insignificantly inhibited at PRBA concentrations below 200 μg/mL. This suggested that there was no link between the cytotoxic effect of PRBA and the inhibition of NO and inflammatory cytokine production. PRBA attenuated Pg-LPS-induced inflammatory responses.

iNOS and COX-2 are well-established as key players in the inflammatory response, with their expression often increased in the inflammatory manifestations of a range of diseases. In this study, Pg-LPS increased the expression of iNOS and COX-2 proteins. Conversely, they were dramatically reduced in the PRBA group. iNOS and COX-2 are involved in the production of NO, which is widely recognized as an inflammatory mediator and regulated by NF-κB[26]. Accordingly, the result observed that increased the expression of iNOS and COX-2 in the Pg-LPS-treated group was accompanied by an overproduction of NO (Figs. 1 and 2). Arg-1, a marker of M2 macrophages, is responsible for tissue regeneration and wound healing[27]. The balance between iNOS and Arg-1 expression is crucial for regulating inflammation and tissue repair to achieve tissue homeostasis[28]. Our study found that Arg-1 expression levels were greater following PRBA therapy, whereas iNOS and COX-2 expression levels were reduced. PRBA are anthocyanins extract from purple rice bran with diverse components. Our previous research team has identified 5 anthocyanidin aglycones and 12 acetylated anthocyanins in PRBA, including cyanidin, peonidin, delphinidin, malvidin, petunidin, C3G and P3G, malvidin-3-glucoside, and so on[11]. Therefore, 2 major anthocyanin components, C3G and P3G, were selected for cell experiments in this study to verify whether anthocyanins play a major role in anti-inflammation. The outcomes demonstrated that both C3G and P3G inhibited the synthesis of TNF-α and IL-6, as well as the expression of the inflammatory gene COX-2.

Although PRBA has shown strong anti-inflammatory effects, its mechanisms remain poorly understood. According to the RNA-seq results, a high proportion of macrophage gene expressions were regulated after PRBA treatment. There were 1 840 DEGs found, of which 1 263 were upregulated and 577 were downregulated. To obtain a more thorough understanding of the precise effects of PRBA on RAW264.7 cells, the RNA-seq data might be integrated with additional bioinformatics analysis, such as GO and KEGG enrichment analyses. Through GO enrichment analysis, PRBA significantly impacted biological processes related to “response to virus”, “regulation of defense response”, “cytokine receptor binding”, and other inflammation-related functions. Based on the results from the KEGG analysis, PRBA inhibited the production of Pg-LPS-induced inflammatory mediators by suppressing the activation of NF-κB and AP-1 signaling pathways, providing crucial insights into the potential molecular mechanisms.

NF-κB is an essential transcription factor that controls several genes during inflammatory responses. It is composed of 2 subunits, p50 and p65[29]. When cells are at rest, NF-κB attaches to the inhibitory protein IκB and stays in the cytoplasm in an inactive form[30]. Extracellular signals cause IκB to become phosphorylated, which makes NF-κB’s nuclear localization sites visible. This causes free NF-κB to move quickly into the nucleus where it binds to particular IκB sequences to trigger the transcription of linked genes[31]. By inhibiting cellular inflammatory responses through the NF-κB pathway, PRBA was found to drastically reduce the nuclear protein p65/Lamin B1 ratio. Correspondingly, it was found that TNF-α was considerably increased and robust activation of p-p65 in the Pg-LPS group that underwent translocation from the cytoplasm to the nucleus, promoting the release of iNOS and COX-2 and the production of inflammatory mediators TNF-α and IL-6.

AP-1 is a crucial transcription factor in RAW264.7 cells, essential for inducing the production of many cytokines and proinflammatory genes, such as TNF-α, IL-6, and COX-2[21]. Upon Pg-LPS stimulation of RAW264.7 cells, AP-1 was released and rapidly moved to the nucleus, activating the transcription of its target cytokine and inflammatory mediator genes. These findings suggested that PRBA had anti-inflammatory properties by preventing NF-κB and AP-1 from being activated, which resulted in less inflammatory mediators such as TNF-α, IL-6, iNOS, COX-2, and NO being produced, it might successfully block the nuclear translocation of AP-1 in Pg-LPS-stimulated RAW264.7 cells. In the future, further relevant *in vivo* studies and clinical applications can be conducted to validate the efficacy of PRBA in animal models, as well as its safety, efficacy, and optimal dosage in human subjects. Through research in these directions, we can gain a better understanding of anti-inflammatory effects of PRBA and provide a scientific basis for translating it into clinical applications.

5. Conclusion

This study demonstrated that PRBA inhibited inflammatory mediators (TNF-α, IL-6, iNOS, COX-2, and NO) produced by Pg-LPS-stimulated RAW264.7 cells. RNA-seq indicated that the NF-κB and AP-1 pathways have a pivotal role in mediating the production of these inflammatory factors. These findings were further confirmed by Western blotting experiments, which showed significant inhibition of the nuclear translocation of NF-κB and AP-1, indicating that PRBA inhibited both NF-κB and AP-1 activation. The results indicated that PRBA might reduce inflammation in RAW264.7 cells induced by Pg-LPS by preventing the activation of NF-κB and AP-1.

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Conflicts of interest

The authors declare no conflict of interest.

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