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The proteomic analysis of anti-inflammatory effects of geniposidic acid in LPS induced THP1 monocytic cells

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ABSTRACT: People's health is adversely impacted by diseases caused by abnormal inflammation; therefore, more effective treatments as well as research into the underlying mechanisms are urgently required. According to our preliminary research, geniposidic acid exhibits anti-inflammatory properties. Even though geniposidic acid has been linked to inflammation and shows promise in the treatment of aberrant inflammatory illnesses, its exact anti-inflammatory mechanism is unknown. To create *in vitro* cellular inflammatory models for 48 h, we employed THP-1 cells treated with PMA (phorbol 12-myristate 13-acetate) and LPS (lipopolysaccharide), which were then treated with geniposidic acid. These models were further validated by ELISA-based detection of the expression of the interleukins IL-1, IL-6, IL-8, and tumor necrosis factor TNF-α. The anti-inflammatory properties of geniposidic acid were investigated using proteomics based on TMT-LC-MS/MS analysis and bioinformatic analysis. Geniposidic acid can act as an anti-inflammatory agent in inflammatory cell models, according to the successfully established THP-1 cell inflammatory models. GO and KEGG analyses further established numerous bioprocesses, molecular activities, and signaling pathways related to the regulation of geniposidic acid. Additionally, western blot analysis confirmed that geniposidic acid can suppress inflammation by lowering the expression of proteins linked to inflammation such as CXCL10, PPM1H, and TLR5. After more thorough mechanistic, animal, and clinical research were included, the current study revealed that anti-inflammatory efficacy of geniposidic acid from the standpoint of proteomics made it a promising medicine to cure and prevent diseases caused by aberrant inflammation.

KEYWORDS: geniposidic acid, phorbol myristate acetate, lipopolysaccharide, TMT-LC-MS/MS, anti-inflammatory mechanisms

INTRODUCTION

Inflammation is a protective reaction involving immune cells, blood vessels, and chemical mediators and is a component of the complicated biological response of human tissues to harmful stimuli such as pathogens, damaged cells, or irritants [1]. Inflammation serves to remove the original source of cell injury, remove necrotic cells and tissues that have been harmed by both the initial result and the inflammatory process, and start the repair process for injured tissues. A large group of disorders known as inflammatory abnormalities underlie a wide range of human diseases [2], seriously affecting people's life and burdening government healthcare system. For instance, sepsis, the most typical cause of death in hospitalized patients, is brought on by an aberrant inflammatory immune response [3, 4]. The worldwide incidence of sepsis is estimated to be 18 million cases per year [5]. Consequently, the effective medications are under urgent need.

Fructus Gardeniae (fruits of *Gardenia jasminoides* Ellis (Rubiaceae), known as "*Zhizi*" in Chinese), is a famous medicinal herb widely distributed in Asia and has been broadly applied in clinical use and food additives in China and other Asian countries [6]. Lines of pharmacology researches demonstrated that Fructus Gardeniae extracts have a wide array of biological activities on inflammation related diseases such as bacterial infections [7, 8], liver injury [9], ligament injury [10], acute pancreatitis [11], gastritis [12], memory impairment [13], and LPS-induced inflammation [14].

Geniposidic acid is one of the representative active ingredients in Fructus Gardeniae [15, 16], which was chosen as one of the quality control markers of this herb [17, 18]. In recent years, researchers have demonstrated that geniposidic acid has better plasma absorption and tissue distribution in pathological states [19]. In our previous study, the metabolic profile of Fructus Gardeniae in human blood and urine was investigated, and geniposidic acid was identified as one of its metabolites [20]. So far, several reports have shown that geniposidic acid has various pharmacological effects including antinociception [21], anti-atherosclerotic [22], anti-hepatotoxity [23], anti-neurodegeneration [24], anti-adjuvant arthritis [25], and especially the anti-liver damage [26]. For instance, Kim et al [27] discovered that geniposidic acid was able to alleviate GalN/LPS-induced liver injury by enhancing antioxidative defense system and reducing apoptotic signaling pathways. Gao et al [28] reported that geniposidic acid had anti-atherosclerotic effects via protecting vascular endothelium and reversing plaque formation. In addition, geniposidic acid could attenuate genipin-induced mitochondrial apop-

tosis without altering the anti-inflammatory ability in KGN cell line [29]. More importantly, geniposidic acid was proven to be the most active anti-inflammation iridoid in *Castilleja tenuiflora* (Orobanco), which was more active than the control indomethacin [30, 31]. However, most of these studies have focused only on the pharmacological effects of geniposidic acid, and data of the anti-inflammatory mechanisms of geniposidic acid is limited.

The discovery of illness biomarkers and the identification of pathways disrupted by the pharmaceuticals have been widely employed in contemporary methods based on 'omics' platforms such as proteomics, to expose the effects and mechanisms of the drugs [32]. Two major analytical techniques including nuclear magnetic resonance (NMR) and high resolution-mass spectrometry (HR-MS) have been widely used in this field [33–35]. In the present study, a combination of cell proteomics and pharmacological strategy based on TMT-LC-MS/MS analysis coupled with multivariate data analysis and pathway analysis was used to study the beneficial effect of geniposidic acid on THP-1 cell inflammation model, which provided a new way for exploring the potential anti-inflammatory mechanism of geniposidic acid and the development of new related drugs.

MATERIALS AND METHODS

THP-1 cell culture

Human monocytic THP-1 cells (ATCC® TIB-202™, China) were cultured in RPMI 1640 (Gibco™, C11875500BT, USA) medium containing 10% of fetal bovine serum (Gibco™, 10099141) supplemented with 10 mM Hepes (Yuanye, S16013, China), 1 mM pyruvate (Sigma, S8636, USA), 2.5 g/l D-glucose (Sigma, G8270), and 50 pM β-mercaptoethanol (Sigma, M3148) in a humidified atmosphere of 5% CO_2 at 37 °C.

THP-1 cell differentiation

We used PMA to induce the differentiation of THP-1 cells, so as to obtain a more optimized inflammatory cell model. The induction of THP-1 cells into mature macrophage-like state was achieved in all experiments by resuspending the cells in culture medium containing PMA (50 nM, Sigma, P1585) for 48 h. After differentiating into macrophages, cells were starved with serum in RPMI 1640 medium containing 2% FBS for 15 h, and then the medium was discarded. LPS (1 μg/ml, Sigma, L2630) was used to stimulate the differentiated THP-1 cells.

Geniposidic acid treatment

In this study, A group of blank THP-1 cells were used as control. B group was set as the THP-1 cells treated with PMA. C group was set as the THP-1 cells treated with PMA + LPS. D group was set as THP-1 cells treated with PMA + LPS + geniposidic acid (320 μM). We set different concentrations of geniposidic acid (the relevant data were not shown), and finally we chose the concentration of 320 μM for the follow-up study). Four groups included control, PMA (50 nM), PMA (50 nM) + LPS (1 μg/ml), and PMA (50 nM) + LPS (1 μg/ml) + geniposidic acid (320 μM).

ELISA (enzyme linked immunosorbent assay)

The A, B, C, and D groups were used in this experiment. THP-1 cells receiving no treatment were used as blank control. Finally, the supernatants of different groups were collected, and the levels of IL1-β (Interleukin-1β), IL8 (Interleukin-8), IL6 (Interleukin-6), and TNFα (tumor necrosis factor α) were measured by ELISA reagent kits (Abcam, ab214025, ab46032, ab46027, and ab181421, UK). The operation process of ELISA was carried out according to the manufacturer's kit instructions.

Proteomic analysis

The sample was suspended in an appropriate amount of SDT cracking solution (protein lysis buffer containing 4% (w/v) SDS and 100 mM DTT), then the resultant mixture was conducted by ultrasound, and the homogenate was boiled for 15 min. After centrifugation at 14,000*g* for 15 min, the supernatant was obtained. The BCA (bicinchoninic acid) method was used for protein quantification. The protein samples were stored at −80 °C. Subsequent processes of acquiring protein samples contain FASP (filter-aided sample preparation), enzymolysis (according to the product manual), and peptide quantification (by Nano Drop 2000). Proteins were quantified and identified by a tandem mass tag (TMT), which is a chemical label used for mass spectrometry. The next main steps are as follows: high pH RP classification (according to the product manual), mass spectrometry analysis, mass spectrometry identification, and data analysis. Briefly, protein extracts were isolated from THP-1 cells (A group was set as the THP-1 cells treated with PMA (induced differentiation). B group was set as the THP-1 cells treated with PMA + LPS. C group was set as THP-1 cells treated with PMA + LPS + geniposidic acid. According to the experimental method described above, the obtained data was analyzed using the data analysis software (Mascot 2.6 and Proteome Discoverer 2.1). Finally, GO enrichment analysis and KEGG pathway analysis were performed [36].

Western blot analysis

There was no difference of inflammatory factors between control (blank) group and PMA group in previous experiments (in fact, PMA could not induce inflammatory process). We planted the THP-1 cells in a 6-well plate, equal to ELISA groups: A group

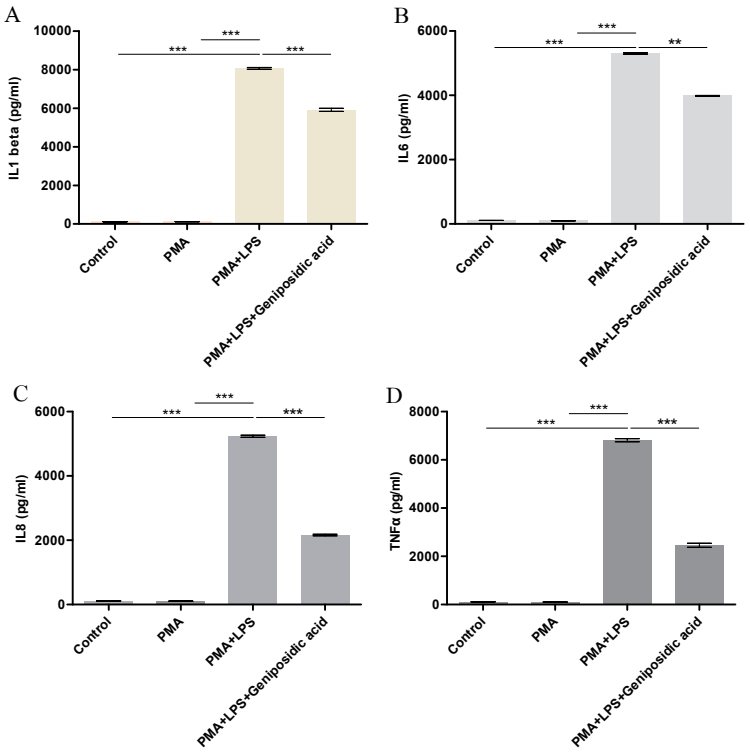

Fig. 1 ELISA results of (A) IL1 beta, (B) IL6, (C) IL8, and (D) TNFα. As shown, geniposidic acid attenuates cellular inflammatory response. Group: Control (Blank), PMA, PMA + LPS, and PMA + LPS + geniposidic acid.

was set as the THP-1 cells treated with PMA (induced differentiation). B group was set as the THP-1 cells treated with PMA + LPS. C group was set as THP-1 cells treated with PMA + LPS + geniposidic acid. We extracted proteins from 3 groups of cells; BCA method was used for quantitative analysis. Then, we used SDS-PAGE to analyze the protein level changes of related genes in the 3 groups. The related genes are CXCL10, PPM1H, and TLR5 (Abcam, ab214668, ab95085, ab168382). GAPDH (Abcam, ab8245) was used as an internal reference protein.

Statistical analysis

In our study, data are presented as mean ± standard deviation. Data processing is mainly based on Student's *t*-test. SPSS 17.0 software was used for analysis, and differences among the groups were assessed using one-way ANOVA. p-values < 0.05 indicated statistical significance. p-values < 0.01 indicated a significant difference.

RESULTS

Geniposidic acid attenuates cellular inflammatory response

Prior to proteomic analysis, we firstly examined whether geniposidic acid can attenuate cell inflammatory response. As shown in Fig. 1, after THP-1 cells were treated with PMA and LPS, the cytokines including IL1-β, IL8, IL6, and TNFα were significantly increased (p=***), demonstrating the success establishment of inflammatory model activated by LPS, which was consistent with the reported literatures [37, 38]. After treatment with geniposidic acid, these cytokines were significantly down-regulated (IL1-β, IL8, and TNFα: p=***; IL-6: p=**), suggesting that geniposidic acid could attenuate cellular inflammatory response.

Differentially expressed proteins were screened and the core proteins were selected

We firstly examined the common differentially expressed proteins. As shown in Fig. 2A, the principal component analysis (PCA) showed there were significant differences among the 3 groups. In Fig. 2B, the heatmap showed that a total of 74 proteins were either up-regulated (red) or down-regulated (blue). To be specific, the expression of CXCL10 (C-X-C motif chemokine 10), PPM1H (protein phosphatase 1H), and TLR5 (toll-like receptor 5) was significantly reduced by geniposidic acid (Table 1).

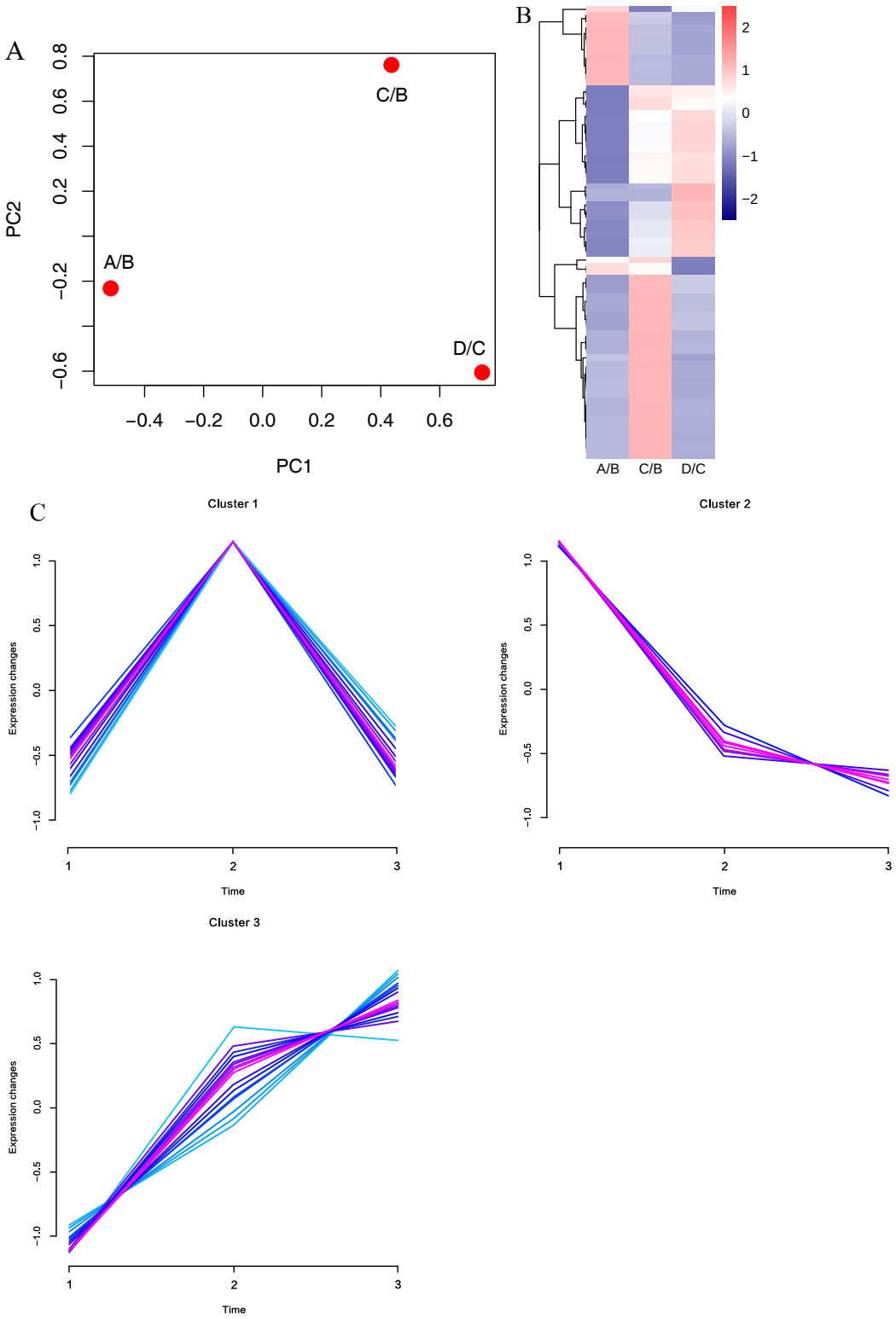

Fig. 2 (A) the principal component analysis (PCA) among the 3 groups (A/B, C/B, D/C). (B) The heatmap showing differentially expressed genes when making pairwise comparisons. Group A: Control (Blank), Group B: PMA, Groups C: PMA + LPS, and Group D: PMA + LPS + geniposidic acid. (C) The patterns of differentially expressed gene expression divided into 3 categories, including cluster 1 (A/B), cluster 2 (C/B), and cluster 3 (D/C).

Table 1 The differentially expressed genes between normal control (A) and Model (B); Model (B) and Model + geniposide (C); normal control (A) and Model + geniposide (C). Green (down-regulated genes). Red (up-regulated genes).

Gene name	A/B	C/B	A/C	Gene name	A/B	C/B	A/C
ZFP36L1	0.461	0.729	0.686	JUNB	0.688	1.471	0.656
CXCL10	0.328	1.582	0.857	ITGAX	0.738	1.255	1.670
DDX58	0.763	1.391	1.363	ITGA6	0.745	1.391	0.727
TLR5	0.842	1.683	0.885	ISG20	0.562	1.391	0.576
TPX2	1.354	0.814	0.704	IL8	0.759	1.391	0.284
TOGARAM1	0.749	0.807	1.654	IFIT1	0.665	1.391	1.527
TMSB15A	1.279	0.748	0.678	IFI44	0.588	1.391	1.764
TDO2	0.451	1.378	0.392	IFI16	0.583	1.391	1.467
SPDL1	2.396	0.827	0.648	HPCAL1	0.694	1.391	1.473
SPC25	1.315	0.823	0.628	HMGA2	0.484	1.391	2.298
SOD2	0.712	1.583	1.536	HEL-S-45	0.650	1.391	1.950
SLFN5	0.777	1.345	1.304	HEL113	0.694	1.391	1.644
SLC16A7	1.521	0.824	0.732	GLNC1	0.515	1.391	0.723
SCARB2	0.650	1.293	1.520	FABP4	0.442	1.391	0.720
RGCC	0.433	0.771	0.704	F5	0.784	1.391	0.706
RAP1GAP2	1.379	1.617	0.694	EPSTI1	0.704	1.391	0.624
PSMB10	1.601	0.795	1.377	EGFL7	1.307	1.391	0.730
PRSS1	0.777	1.229	1.332	DUT	1.323	1.391	0.590
PRG1	3.593	1.317	0.605	DOCK4	0.755	1.391	1.445
PINLYP	0.516	1.707	0.426	PPM1H	0.628	1.473	0.824
PIAS1	1.312	1.200	0.707	CPA4	1.322	1.391	1.663
PBEF1	0.762	1.357	1.488	COL6A1	0.579	1.391	0.753
PALLD	0.559	1.461	0.665	CEBPB	0.566	1.391	0.546
OAZ2	0.517	1.582	0.446	CDCA1	1.475	1.391	0.725
NRP2	0.776	1.573	0.582	CDC45	1.512	1.391	0.686
NR4A3	0.648	1.274	0.569	CD9	0.303	1.391	0.686
NPL	0.764	1.310	2.150	CD209	0.504	1.391	0.416
NOGOC	0.650	1.394	0.694	CCNA2	1.611	1.391	0.638
NCK1	0.597	2.156	0.672	CCL3L1	0.493	1.391	0.218
MX1	0.590	1.240	1.615	CCL2	0.747	1.391	0.168
MSR1	0.545	1.332	0.641	BTG2	0.747	1.391	0.695
MAFB	0.446	1.289	0.601	BCL2A1	0.608	1.391	0.573
LIG1	1.598	0.812	0.724	ATP2B1	0.757	1.391	1.369
LAMP5	1.583	0.709	0.650	APOH	0.595	1.391	0.709
KRT14	0.780	0.733	5.233	APOE	0.582	1.391	0.730
KRT1	0.777	0.810	2.963	APOC1	0.483	1.391	0.501
KANK1	0.628	1.276	0.583	AMPD3	0.677	1.391	0.501

Patterns of protein expression

The patterns of protein expression were divided into 3 categories, including cluster 1(A/B), cluster 2(C/B), and cluster 3(D/C). A: control group, B: PMA (50 nM) group, C: PMA (50 nM) + LPS (1 μg/ml) group, D: PMA (50 nM) + LPS (1 μg/ml) + geniposidic acid (320 μM) group. As shown in Fig. 2C, the differentially expressed proteins in cluster 1 showed a down-up-down trend, in cluster 2 showed an up-down-down trend, and in cluster 3 showed a down-up-up trend.

GO enrichment analysis

Furthermore, the GO enrichment analysis of each category was performed respectively. As shown in Fig. 3, in cluster 1, the major bioprocess is cellular process (23%), the major molecular function is binding (56.2%), and the major signaling pathway is angiogenesis (31.1%). In cluster 2, the major bioprocess is

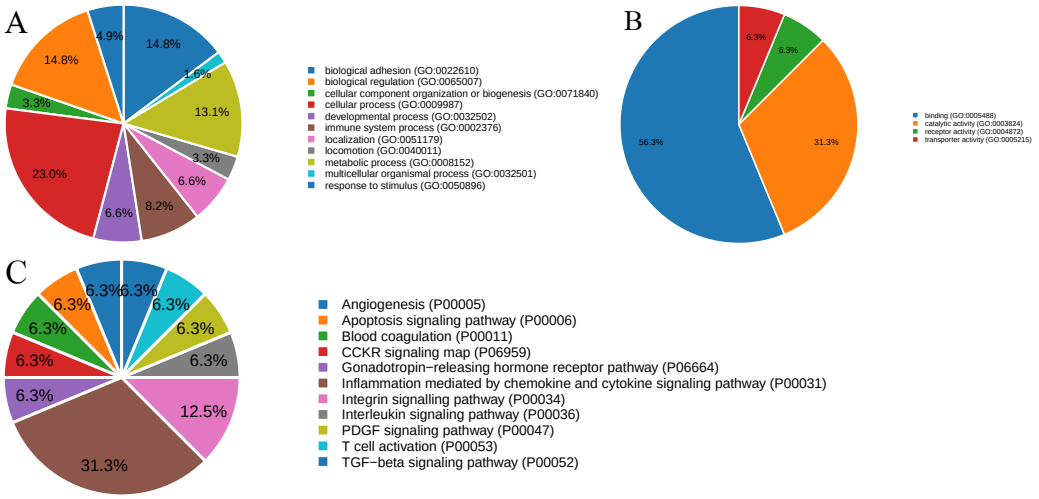

Fig. 3 The GO enrichment analysis including bioprocesses, molecular functions, and signaling pathways in cluster 1. (A) The major bioprocesses. (B) The major molecular functions. (C) The major signaling pathways.

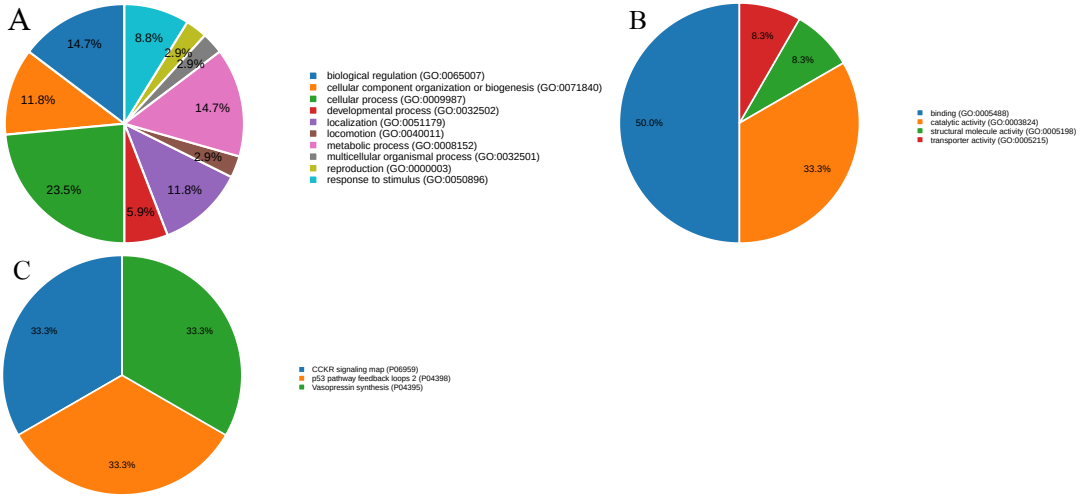

Fig. 4 The GO enrichment analysis including bioprocesses, molecular functions, and signaling pathways in cluster 2. (A) The major bioprocesses. (B) The major molecular functions. (C) The major signaling pathways.

cellular process (23.5%), the major molecular function is binding (50.1%), and the major signaling pathway is CCKR (cholecystokinin receptor) signaling map (Fig. 4). In cluster 3, the major bioprocess is metabolic process (40.0%), the major molecular function is binding (38.1%), and the major signaling pathway is interleukin signaling pathway (16.7%), (Fig. 5). The results of other GO enrichment analyses were also shown in Fig. 5.

Signaling pathway network analysis

Finally, we analyzed the signaling pathway network and found that IL-8 was the core target participating in many pathways. As shown in Fig. 6, it mainly participates in 4 signaling pathway networks including regulation of wound healing, PERK (PKR-like ER kinase)-mediated unfolded protein response, glycerolipid catabolic process, and brown fat cell differentiation. According to the reports of related literatures, IL-8 is involved in these 4 signaling pathways, and the conclusion of this study on IL-8 is consistent with the literature reports.

Potential mechanism exploration

Additionally, the protein expression of differentially expressed proteins was verified *in vitro*. As shown in Fig. 7, the expression of CXCL10, PPM1H, and TLR5 of THP-1 cells treated with PMA + LPS was significantly higher than those of the THP-1 cells treated with PMA (fold change). Interestingly, the expression of these 3

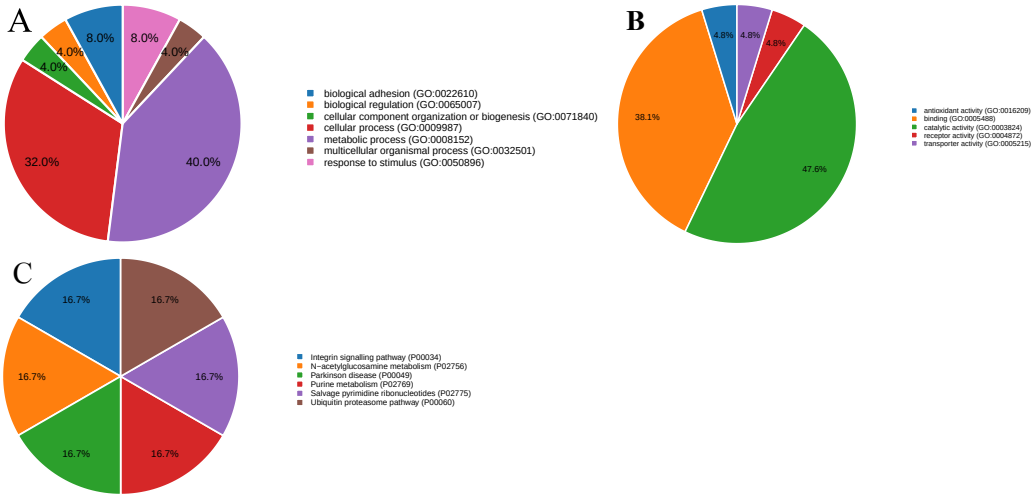

Fig. 5 The GO enrichment analysis including bioprocesses, molecular functions, and signaling pathways in cluster 3. (A) The major bioprocesses. (B) The major molecular functions. (C) The major signaling pathways.

genes in THP-1 cells treated with PMA + LPS + geniposidic acid was obviously reduced compared to the model group (fold change). These results suggested that geniposidic acid could protect THP-1 cells against abnormal inflammation via the regulation of CXCL10, PPM1H, and TLR5.

DISCUSSION

Inflammatory abnormalities are a large group of disorders that cause many human diseases. The immune system is often involved with inflammatory disorders, demonstrated in both allergic reactions and some myopathies, with many immune system disorders resulting in abnormal inflammation [2]. The abnormal inflammation induced diseases seriously affect human health. However, the relevant mechanism is still unclear, and more effective therapeutic drugs are needed. Therefore, it is very important to study the molecular mechanism of cellular inflammation. For instance, the latest research found that KLF7 can promote LPS induced apoptosis, inflammation, and oxidative stress in H9c2 cells by activating NF-κB pathway. Knocking down KLF7 could be a therapeutic method to treat sepsis (diseases related to abnormal cell inflammation) [39]. KLF7 could become a potential drug target for the treatment of sepsis in the future. In this study, we selected geniposidic acid as a potential therapeutic drug for treating diseases induced by abnormal inflammation and performed proteomic analysis to investigate its potential mechanism.

Proteomics is a large-scale study of proteins [40], and one major development coming from the study of human genes and proteins has been the identification of potential new drugs for the treatment of diseases [41]. Moreover, protein-protein interactions were significantly involved in the development of diseases. Interaction proteomics become important, attractive, and effective in identifying binary protein interactions, protein complexes, and interactome (protein-protein interaction network in a specific organism) [42].

In this study, the GO enrichment analysis of clusters 1, 2, and 3 revealed that they shared the common bioprocesses and molecular functions. The common bioprocesses included cellular process, biological regulation, and response to stimulus. The common molecular functions included binding and catalytic activity. However, there was no common signaling pathway found. Moreover, the signaling pathway network analysis discovered that IL-8, which is associated with inflammation, mainly participated in 4 intersecting signaling pathway networks, including regulation of wound healing, PERK-mediated unfolded protein response, glycerolipid catabolic process, and brown fat cell differentiation. At the end of this study, we further verified the differentially expressed proteins, including CXCL10, PPM1H, and TLR5. Among the 3 proteins, CXCL10 and TLR5 have close releationship with inflammation. CXCL10 is secreted by several cell types in response to IFN-γ. In addition, CXCL10 has been attributed to several roles such as chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells, which were major immune cells of inflammation [43]. TLR5 is a member of the toll-like receptor (TLR) family. The TLR family plays a fundamental role in pathogen recognition and activation of innate immunity. The activation of this receptor mobilizes the nuclear factor NF-κB and stimulates TNF-alpha production, which is another important inflammatory cytokines [44]. PPM1H was found in this study to be one of the target proteins for geniposidic acid to play an anti-inflammatory role. Through the analysis

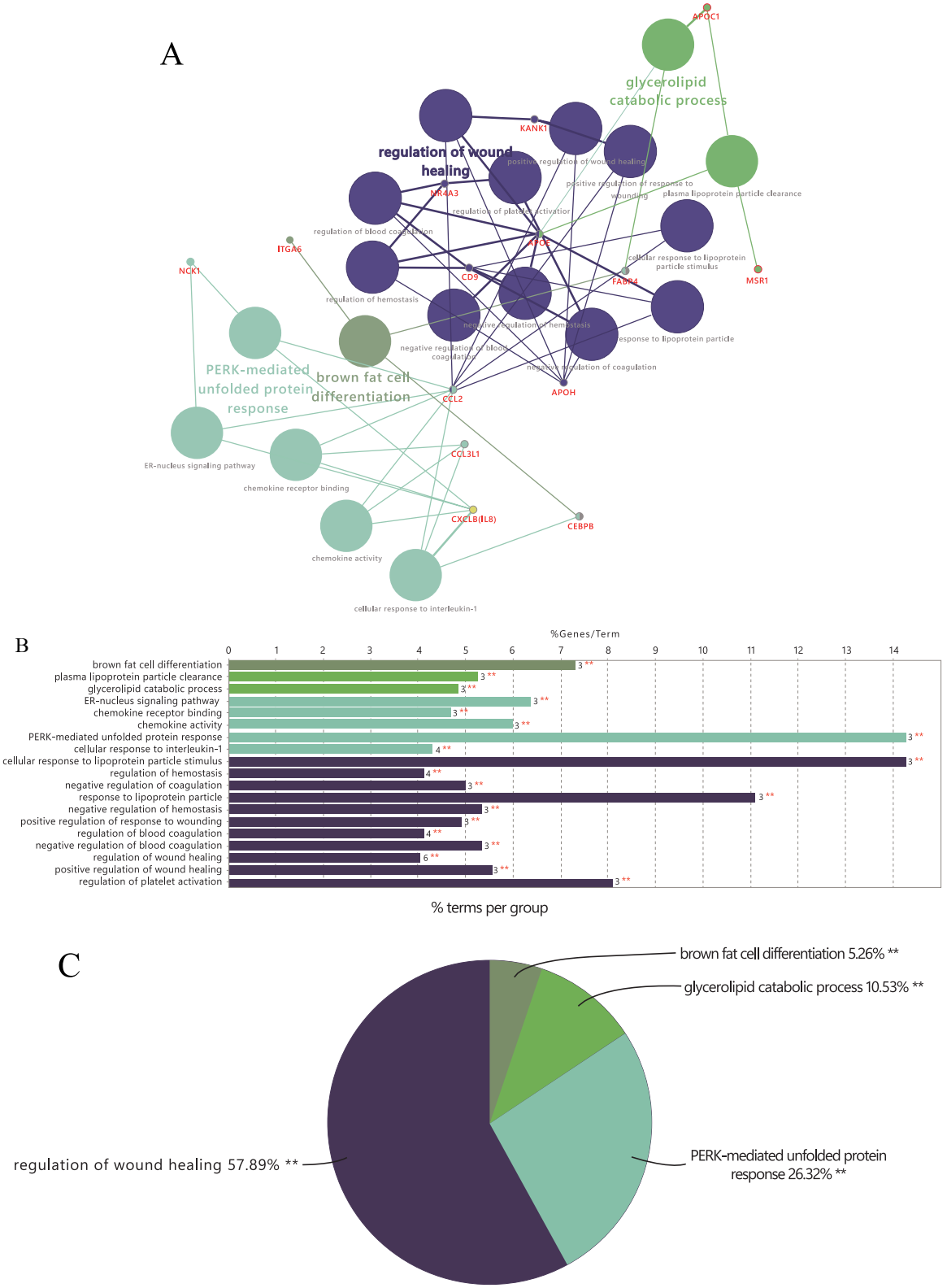

Fig. 6 Intersecting signaling pathway network analysis. (A) Signaling pathway network, (B) % genes/term, and (C) % terms per group.

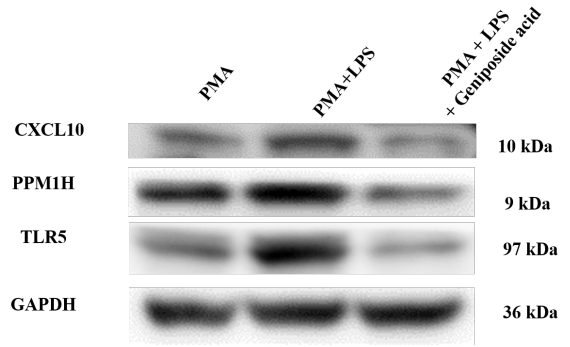

Fig. 7 The protein expression of CXCL10, PPM1H, and TLR5 of THP-1 cells treated with PMA, PMA + LPS, and PMA + LPS + geniposidic acid.

of the IntAct database, it is found that PPM1H can directly bind and interact with 48 protein molecules, and most of them have been clearly involved in the cellular inflammatory process. PPM1H, as a dephosphorylase, infers that PPM1H may participate in the cellular inflammatory process through the dephosphorylation of binding proteins, but this requires our subsequent experiments and verification. In fact, IL-8 and CXCL10 are markers of cellular inflammation, showing a trend of co-expression. TLR5 mediated p38 MAPK activation regulates the expression of IL-8 through a post-transcriptional mechanism. At present, there is no research to explain the relationship between TLR5, CXCL10, and PPM1H. However, according to the description of our discussion part and the results we have observed so far, IL-8, CXCL10, TLR5, and PPM1H may have a network of mutual regulation, but this requires subsequent experiments and demonstration. In summary, the current study demonstrated that geniposidic acid could play the anti-inflammatory roles via inhibiting CXCL10, PPM1H, and TLR5 and impressing a variety of protein interactions, therefore making it a promising approach against abnormal inflammation induced diseases.

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