

18β-Glycyrrhetic Acid Alleviates *P. multocida*-Induced Vascular Endothelial Inflammation by PARP1-Mediated NF-κB and HMGB1 Signalling Suppression in PIEC Cells

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Background: At present, the treatment and prevention of *Pasteurella multocida* infections in pigs mainly rely on antibiotics and vaccines, but inflammatory injury cannot be eliminated. The compound 18β-glycyrrhetic acid (GA), a pentacyclic triterpenoid extracted from *Glycyrrhiza glabra* L. root (liquorice) and with a chemical structure similar to that of steroidal hormones, has become a research focus because of its anti-inflammatory, antiulcer, antimicrobial, antioxidant, immunomodulatory, hepatoprotective and neuroprotective effects, but its potential for the treatment of vascular endothelial inflammatory injury by *P. multocida* infections has not been evaluated. This study aimed to investigate the effects and mechanisms of GA intervention in the treatment of vascular endothelial inflammatory injury by *P. multocida* infections.

Materials and Methods: Putative targets of GA intervention in the treatment of vascular endothelial inflammatory injury by *P. multocida* infections were identified using network pharmacological screening and molecular docking simulation. The cell viability of PIEC cells was investigated via the CCK-8 assay. The mechanism of GA intervention in the treatment of vascular endothelial inflammatory injury by *P. multocida* infections were investigated using cell transfection and western blot.

Results: Through network pharmacological screening and molecular docking simulation, this study found that PARP1 may be a core target for GA to exert anti-inflammatory effects. Mechanistically, GA alleviates *P. multocida*-induced vascular endothelial inflammation by PARP1-mediated NF-κB and HMGB1 signalling suppression.

Conclusion: These findings, for the first time, demonstrate the potential therapeutic relationship among GA, PARP1 and inflammatory injury, providing a candidate drug, therapeutic targets and explanation for treating vascular endothelial inflammatory injury caused by *P. multocida* infection.

Keywords: network pharmacology, GA, PARP1, inflammatory injury, *P. multocida* infection

Introduction

Pasteurella multocida is a zoonotic pathogen with the ability to infect a wide range of hosts including humans as well as domestic, wild and companion animals.^{1,2} Infection with *P. multocida* is responsible for significant morbidity and mortality in humans and animals.^{1,3} The pathogen is one of the culprits causing major economic losses in the pig industry worldwide because of its persistently high prevalence: 8% prevalence with pneumonia or progressive atrophic rhinitis (PAR) between 2003 and 2007 in China,⁴ 3.4% prevalence of 44,175 samples between 2013 and 2017 in China,⁵ 15.6% prevalence of 33,813 isolated swine respiratory cases between 2003 and 2010 in the United States,⁶ 15.8% prevalence between 2009 and 2010 in South Korea,⁷ 16.8%

prevalence between 2008 and 2016 in Korea.⁸ In clinically diseased pigs, most *P. multocida* strains are characterised as capsular type A and D, causing respiratory disorder in pigs, such as PAR, pneumonia, pleurisy and septicaemia.^{9–11} The haemorrhagic pneumonia caused by *P. multocida* infection suggests severe injury to the vascular endothelial cells at the site of pathogenesis and is accompanied by increased vascular endothelial permeability. At present, clinical prevention and treatment of *P. multocida* infection are mainly based on the application of antimicrobials and vaccines,^{9,12} which do not eliminate the vascular endothelial inflammation injury caused by *P. multocida* infection but also cause the development of drug resistance. In addition, antimicrobials, glucocorticoids or non-steroidal anti-inflammatory drugs often cause adverse effects such as bacterial endotoxin release, immune suppression or gastric ulcers. Therefore, elucidating the molecular mechanisms of vascular endothelial inflammation injury caused by *P. multocida* infection and developing adjuvant therapies or effective drugs to control inflammation *P. multocida* infection are of great scientific interest and application to reduce the harm of *P. multocida* infection.

Poly (ADP-ribose) polymerases-1 (PARP1) is a direct predictor of inflammatory diseases, and its central role in inflammation is the regulation of nuclear factor-kappa B (NF- κ B) activation and high mobility group box 1 (HMGB1) secretion.^{13,14} The PARP1 itself promotes the transcription of NF- κ B-dependent genes of inflammatory cytokines, such as interleukins 6 and 1 β (IL-6, IL-1 β) and tumour necrosis factor α (TNF- α).¹⁵ Moreover, in in vivo models, PARP1 is involved in the regulation of inflammation in bacterial infections. In a model of *S. typhimurium*-induced murine colitis, loss of PARP1 reduced NF- κ B-mediated proinflammatory gene expression, contributing to the alleviation of intestinal inflammation.¹⁶ The PARP1 mediates bacterial endotoxin-induced HMGB1 release from macrophages, and PARP1-dependent translocation of HMGB1 from the nucleus to the cytoplasm can increase the activation of cellular inflammatory pathways.¹⁴ However, the effect of *P. multocida* infection on PARP1 expression and whether PARP1 alleviates *P. multocida*-induced vascular endothelial inflammatory injury by regulating NF- κ B and HMGB1 remain unclear.

According to traditional medicine, *Glycyrrhiza glabra* L. root (licorice) is one of the oldest and most widely used herbs in the world and applied to moisten the lung and relieve cough and toxicity, dispel phlegm, promote meridians and harmonise the nature of other herbal medicines.¹⁷ The compound 18 β -glycyrrhetic acid (GA), a pentacyclic triterpenoid extracted from licorice and with a chemical structure similar to that of steroidal hormones, has become a research focus because of its anti-inflammatory, antiulcer, antimicrobial, antioxidant, immunomodulatory, hepatoprotective and neuroprotective effects.¹⁸ In a previous study, GA inhibited *Staphylococcus aureus*-induced RAW264.7 cell inflammation by inhibiting the activation of NF- κ B and the expression of HMGB1;¹⁹ in addition, it has a protective effect on *Helicobacter pylori*-infected gastric mucosa through alleviating the expression levels of inflammation-related cytokines (IL-1 β , TNF- α).²⁰ However, the protective role of GA against *P. multocida*-induced vascular endothelial inflammatory injury via PARP1 is still largely unclear.

In this study, drug target prediction and experimental validation were used to explore the mechanism by which GA alleviates vascular endothelial inflammatory injury caused by *P. multocida* infection through regulating the PARP1-NF- κ B/HMGB1 pathway, with the aims to prove that GA has potential to serve as a novel antibiotic alternative and to provide a basis for clinically alleviating vascular endothelial inflammatory injury in *P. multocida*-infected piglets.

Materials and Method

Reagent and Chemicals

The GA (CAS NO. 471-53-4; Catalog # HY-N0180) was purchased from MedChemExpress, USA.

Network Pharmacological Analysis

The potential protein target of GA was identified by the drug target database, such as PharmMapper,²¹ SwissTargetPrediction²² and TCMSP.²³ The targets of vascular endothelial injury were obtained from the PubMed database (<https://pubmed.ncbi.nlm.nih.gov>) and the GeneCards database (<https://www.genecards.org>). The Veen software (www.bioinformatics.com.cn) was employed to select the common target of the potential protein target of GA and the targets of vascular endothelial injury in pigs. The core target was selected by the protein-protein interaction (PPI) network of the String database (<https://cn.string-db.org>), using the Cytoscape 3.7.2 software.²⁴ Subsequently, GO enrichment analysis and the Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis were

used to analyse the functions and the involved signalling pathways of core genes in the database for annotation, visualisation and integrated discovery (DAVID) (<https://david.ncifcrf.gov>), with the species limited to “*Sus scrofa*”. Finally, the results of GO and KEGG analysis were visualized using a bioinformatics tool (www.bioinformatics.com.cn).

Molecular Docking

The SWISS-MODEL (<https://swissmodel.expasy.org>) was employed to construct the protein structure homology-modelling of PARP1.²⁵ Subsequently, molecular docking between PARP1 and GA was conducted using the software SYBYL-X 2.0. After molecular docking, the results were visualised by the PyMOL software.²⁶

Bacterial Strains and Cell Culture

Pasteurella multocida strain HB03 (serogroups/genotypes A) was kindly provided by Professor Bin Wu and Dr. Zhong Peng (Huazhong Agricultural University, Wuhan). Strain HB03 was cultured in trypticase soy broth medium (Hopebio, China) supplemented with 5% bovine serum (NEWZERUM Ltd, New Zealand, Catalog # NCS-500) at 37°C for 8–12 h.

Porcine vascular endothelial cell line (PIEC) cells were kindly provided by Professor Hongkui Wei and Professor Menghong Dai at Huazhong Agricultural University (Wuhan, China). For culturing PIEC cells, RPMI medium modified (Cytiva, USA, Catalog # SH30809.01) with 1% penicillin-streptomycin solution (Gibco, USA, Catalog # 15070063) and 10% foetal bovine serum (PAN, Germany, Catalog # ST30-3302) was used. The cultures were cultured in a humidified incubator set at 37°C and 5% CO₂. After inoculating the PIEC cells in the cell culture plate for approximately 12 h, the cultures were preincubated with GA for 2 h and then co-treated with strain HB03 (MOI = 50) for another 10 h. All experiments were performed at least in triplicate on three separate occasions.

Cell Viability Assay

The cell viability of PIEC cells was investigated via the CCK-8 assay. For this, PIEC monolayer cells in each well of a 96-well plate were incubated with a series of GA (5–160 µg/mL) for 24 h, followed by the addition of 10 µL CCK-8 solution (Vazyme, China, Catalog # A311-02) into each well of the plate for 2 h. The absorbance of formazan was measured at 450 nm using a microplate reader (Spectra MIX i3x, Molecular Devices, China).

Cell Transfection

The PIEC cells were transiently transfected using the lipo8000™ transfection reagent (Beyotime, China, Catalog # C0533). For the PARP1 overexpression plasmids, PIEC monolayer cells in each well of a 6-well plate were transfected with 125 µL Opti-MEM (Gibco, USA, Catalog # 31985070) diluted in 3.75 µg of DNA (pcDNA3.0-PARP1 or pcDNA3.0) and 3.75 µL of lipo8000™ transfection reagent. For the RNAi assay, PIEC monolayer cells in each well of a 6-well plate were transfected with 125 µL Opti-MEM diluted with 7.5 µL of siRNA (siRNA of PARP1 or negative control siRNA (GenePharma, China) and 5 µL of lipo8000™ transfection reagent.

Western Blot

Challenged PIEC cells were lysed in RIPA buffer (Beyotime, China, Catalog # P0013B) with a protease and phosphatase inhibitor cocktail (Beyotime, China, Catalog # P1045), sonicated and centrifuged at 10,000 × g for 10 min at 4°C. The protein concentration of the supernatant was measured by the enhanced BCA protein assay kit (Beyotime, China, Catalog # P1010). Briefly, 20 µg of protein from each sample was separated via 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Sangon Biotech, China, Catalog # C671102-0125) and then transferred to polyvinylidene difluoride membranes (Millipore, USA, Catalog # SE4M039I09). After being blocked with 5% BSA in tris-buffered saline with Tween 20 for 2 h at room temperature, the blots were incubated overnight with the primary antibodies-anti-PARP1 polyclonal antibody (Proteintech Group, China, Catalog # 13371-1-AP, 1:1000 dilution) anti-β-actin polyclonal antibody (Proteintech Group, China, Catalog # 20536-1-AP, 1:1000 dilution), anti-phospho-NF-kappa-B transcription factor p65 monoclonal antibody (p-NF-κB p65) (Thermo Fisher Scientific, USA, Catalog # MA5-15160, 1:1000 dilution), anti-IL-1β polyclonal antibody (Abclonal, China, Catalog # A1112, 1:1000 dilution), anti-IL-18 polyclonal antibody (Abclonal, China, Catalog # A16737, 1:1000 dilution), anti-HMGB1 polyclonal antibody (Abclonal, China, Catalog # A2553,

1:1000 dilution) and anti-NF-κB p65 polyclonal antibody (Abclonal, China, Catalog # A11204, 1:1000 dilution). After washing, the blots were incubated with secondary anti-IgG antibody (Abclonal, China, Catalog # A11204, 1:5000 dilution) and then visualised with enhanced chemiluminescence reagents (Abclonal, China, Catalog # RM00021) by the FluorChem E (ProteinSimple, China). All western blots were densitometrically quantified using the ImageJ software (ImageJ, NIH).

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 for Windows. All results were presented as mean ± standard deviation. Results were statistically analysed by one-way ANOVA followed by Duncan’s post hoc analysis. Statistical significance was set at $p < 0.05$, and a value of $p < 0.01$ was considered to be strongly significant.

Results

Identification of the Core Targets from Network Pharmacological Analysis

To explore the potential core targets of GA for attenuating *P. multocida*-mediated vascular endothelial inflammatory injury, we explored the core targets from the target database. Target database analysis yielded 216 and 749 targets for GA and vascular endothelial injury, respectively (Figure 1A and B, Supplementary Table 1). The 69 common targets of GA

A

TYMP	MB	YAP1	VHL	STXB1P	SERPINC3	BMP8	PROM1	MAF	EDNRA	MMP9	HMGCR	ESM1	PTX3	ACTC1	KCNMA1	C5	CSPG4	AHSP	STAB1	CDKN3	CREBBP	CD14	LIPC	INS	PLA2G4A	FAS
LCN2	PTGS6	FOXO1	PLA2G2A	PSEN1	NDP	TNFSF11	NOTCH4	FLNA	EFNB2	ROCK2	EGFR	WNT5A	CD4	MAPK3	FOS	ARNT	ARID1B	SERPINF1	SULT1A3	HSPB1	NRG1	PTX2	PKNOX3	SOD3	RBP4	GNA14
RAP1A	CYBA	SERPINA3	PAX8	CDK4	TGFB1	GPT	BDNF	MTND1	COX6	MYC	NTRK2	MBL2	AGTR1	TTR	MDK	PTK2	ECE1	CLDN5	ADCY10	ABCC8	COND1	OSM	SETD2	CAT	GAT3A	CAMTA1
PCDH12	EPO	STAT1	CXCR2	PLA2G7	TGFBF1	IL18	COL1A1	PCNA	PTGS1	SDHD	CDK2	XBP1	BMP4	FOGR2A	UBAD1	CD248	IL6	PDGFRA	F2	ROBO4	PLAT	MME	TGFBF2	ABCC1	KRT3	GP6
PTX2B	NRPB	PLPP3	TSC2	PRK02	EPH4	CFH	AKT2	KRT8	RAC1	COMP	TCF4	MAPK14	BGN	SPTAN1	MIF	JAK2	RTN4	TJP1	TIE1	NOS2	CTLA4	ALB	NOD2	TERT	CYBB	SDHB
KRT11	GASR1	PKD1	BMP2	FCGR2B	DPH4	PGF	IFNG	ZFN4	PDGFA	NES	PLPAP	RHOB	TAGLN	HPRT1	CYCS	MPD2	CHUK	IL1RN	KIT	IL13	SYNGAP1	ANKA1	THPO	CHAT	CLB2	CAV1
CIA	NGFR	NOS1	SIRT1	HIF1A	RUNX2	LIF	ABCA1	NOTCH1	SLC22A5	JUP	ADAMTSL1	FN1	LEPR	PRKD2	PRIP	EDG	LMNA	SCARB1	ADGF1	ITGA6	RBI1	F11R	COL4A2	HAVCR1	CASP3	SEMA3A
ESR2	MEF2C	PROK1	MFN2	IL4	GP1BA	NRP2	ELAVL1	S100B	EPAS1	CDKN1B	SMARCA4	APP	AGT	GPX1	CD40LG	HP	MYH11	NTRK1	HLA-B	ANTXR1	ANKX4	THBS1	IRF5	SHC1	TNFSF15	KRT18
SHH	TNFRSF1A	PROC	IGFBP3	PXN	MGP	CYP11B1	CDH5	CETP	IL1R1	VCAM1	APLN	SP1	CLU	STAT3	ABL1	KCNK3	CASP9	CERCAM	TP53	NPPA	PDGFB	TREX1	SOD1	EDL3	IL2	ACVRL1
FGF7	KRT7	TGFB1	FBG	F2R	PON1	REN	NGF	PEAR1	NEFL	APOE	V5X1	SELE	CCM2	CYSLTR2	ENO2	FLT4	PARP1	SDC1	LGALS3	PGR	SMAD2	WVTR1	ANG	GRB2	HSPA5	EDF1
GFAP	FOXO3	HTR2A	CTNND1	PLA2G6	POMC	SARS1	KITLG	GCH1	COL8A2	PRSS33	FLT3	L1B	DDAH1	TLR2	CTSB	ITGAM	GNAQ	ABO1	HSPA1A	LDLR	CALCR	PK3R2	SMAD3	CASP9	PRKCD	IL3
NINJ1	PLAUR	GJA1	CAV2	KAT5B	TGFA	RARA	AIF1	VEGFA	TNFRSF18	ADRB2	S1PR2	F5	FGA	GH1	APOC1	AGTR2	EFEMP2	LEMD3	HDAC4	FZRL1	VIP	TGFB3	LBP1	FU1	PF4	TNFSF12
SCN9A	PDE3A	GSR	CFI	PPARA	CREB1	GATA4	PXDN	COL18A1	PRKCB	CXCR4	LEP	AGER	RARRS2	CST3	MBP	MMP2	TIMP2	AGBL1	ITGB1	CSF1	LPAR1	DCN	NFE2L2	GAPDH	CYP2C19	SREBF1
MPO	ZNF498	SERPINE1	CHST6	CDC42	MMIP4	TMP1	HMBG1	IL5	SLC6A4	IL8	HRAS	SLC2A1	DUSP29	SLC4A1	CALR	RHOD	ITGB3	GNF	NF1	IGFBP7	INSR	RHO	GRIN1	B2M	ADAM17	CX3CR1
ADMTS13	F7	ADN	ITGA2B	GRIN2B	PPAR0	RETREG1	PLXDC1	APOA1	COL4A4	CD34	AXL	ITGA2	ZEB1	BCL2	ENPP1	DRD2	PDCC10	PK3C2A	PP3CA	IGF1	FGG	OLR1	PRKQ1	CDH2	MAP2K1	TSC1
SMTN	MEFV	TAM	PROX1	EDNRB	SRC	FOXO1	MKG1	RET	AT1RP	TF	NOF	HSPD1	SST	PTEN	BMPR2	QPRT1	DLI1	PRKX1	CRP	NOTCH3	ADA	IGF1R	PKCQ1	ODC1	CEACAM1	F10
ADP1	MTOR	HRH1	CCRS	PIK3R1	AKT3	SP1	FYN	NR4A1	TLR4	TNFAIP3	CD36	HSPM4	SOO2	JUN	MMP3	HSPG2	NPPC	SELPLG	COL5A1	APP	CALCA	FGFR1	TH	ROCK1	MBNL1	KL
IL1A	BMP7	HLA-G	CCL5	MYLK	ITGAL	CCM1	F9	NLRP3	INPP5E	RASA1	APOB	ICAM2	CD46	LMX1B	ATP1B1	CCN1	GPA	ITGA3	THBD	PTGS2	ARG1	BDKRB2	CD47	ANGPTL4	ENG	
EGR1	GAS6	LPA	NR1H2	ALOX12	MUC1	COMT	NR3C2	DDAR2	CDK1	SPARC	ELANE	ACE2	PLG	POG2	AKR1B1	SIPR3	NFKB1	FGF2	HBB	RHOA	TPSANT2	HLA-DQB1	MMP12	TRF4	WT1	CXCL10
PK3CB	PECAM1	IRF3	SFTPC	NOTCH2	ELN	LAMC1	VASH1	PLAU	PROCR	NOS3	SMAD4	IGF2	DGKE	CCL11	CACNA1A	COL4A5	VEGFD	MET	ILF2	CXCL8	FOXO3	RPS6KB1	PRO2	NR3C1	CD99	CXCR3
F3	RAF1	ACP1	JAG1	NFKBIA	BMX	DUSP1	NRX4	CSF3	PSEN2	FBN1	LPL	C3	RETN	IRAK1	ADAM10	ANGPT2	GHR1	MAPT	EDN1	CBS	LRR6	UCHL1	CDH1	MAPK1	HFE	HLA-A
IL2RA	ESAM	BCOR	PALS1	FOXO1	CCL3	MAPK10	ADH	PDN	BRAF	NKIK7	PRL	CXCL9	NOX1	DPYD	GLMN	NTSE	CD55	HEY2	CCR2	TGFB2	CAMK2G	PRKN	ANKX4	CCL2	SELL	PP1A
PTPN11	ADA2	MYD88	CP	MYO88	HTRAI1	FGF1	GNM1	IL6R	PRKDI1	NR4A3	ITGA5	CXCL12	COL1A1	SIPR1	GATA6	IL17A	PTPRB	XDH	MAP8B	MYOCD	VEGFA	SLC17A5	ADP4	PRTH3	SYK	HMG2
VM	SOSTM1	F13A1	TFPI	NPY	ITGA4	CNR1	PRK1	COL4A1	BIRC5	PTN	BSG	PROX3	FGFR2	RAP1B	ADIPOQ	COL4A3	TKT	APLN	TGFB1	TBX1	ANGPT1	HSP90AA4	PGF	AMPI	NRS	MDM2
EP300	PRKCA	ATM	PM52	SELP	IFNA1	TFE3	MDCP2	PTPN1	ICAM1	MAP3K5	BAX	NRP1	CDKN2A	COX5A	OCLN	LYVE1	VTN	CXCL1	CDH13	IL33	FLT1	MTHFR	ERBB2	F8	ACTA2	ETS1
PK3CA	FBLN5	TNF	CXCL1	COL1A2	JAM3	TNC	KRT20	MMP1	VCL	TFRC	IL15	HBEF	ACE	IL10	KDR	ANPEP	TEK	ELMO2	MADCAM1	CXCL2	ITGAV	DLL4	COL3A1	CTNNA1	ZNF469	AOC3
FABLG	ACHE	EPRS1	TIMP3	RPL5	CSF1R	EGF7	LIX	ADORA2A	CD40	CCR8	TNFRSF114	SPEG	CDKN1A	RYR1	RELA	CSF2	HPSE	BGLAP	DIPK1A	PRKCC	IRS1	IGFBP1	HMOX1	CCN2	BCL2L1	ALOX5
C8orf72	DNMT1	ITGB2	SLC2A10	VWF	CFLAR	KLF4	PDGFC	FOF3	NAMPT	ITGB4	JAM2	TNFSF10	TNFRSF11B	VEGFB	GLA	ESR1	PDGFRB	GATA2	CD44							

B

CTSV	PDE3B	AMY1A	HSP90AA1	HMGCR	PIPA	TNNC1	FECH	SORD	STS	BACE1	MAPK10	PARP1	RARA
METAP2	ABO	HNF4G	CTNNA1	SI100A9	PDPK1	PLA2G2A	CSNK2A1	ESR1	FABP7	BLVRB	PAH	PLAU	CASP3
AKR1C1	DFEP1	DKC	ADAM17	ESRRG	XIAP	PPSPC	CLPP	MAOB	GC	HSP90AB1	BRAF	CES1	ADH1B
DHODH	RXRA	PIM1	MMP2	CBR1	LSS	SOD2	MMP13	MAPKAPK2	F7	PADH	MDM2	ACHE	ACADM
RARB	PRKCH	SULT1E1	RXR8	PDE4B	AR	PTPN1	JUP	CASP7	NQO2	AGXT	AKR1C2	FGFR2	BCH
ANKX5	MAPK14	MAP2K1	UCK2	ALB	CCNA2	NR3C1	APOA2	PRKACA	ADK	JAK2	CYP2E1	F2	PLEKHA4
MTAF	CYP2C8	THRB	CSK	TTR	IL2	REN	AKR1B1	RARG	AURKA	PRKCC	PTPN2	PAK5	ITK
BCAT2	FABP4	DUSP6	TYMS	PPARG	SETD7	CA1	NOS3	FKBP1A	BMP2	CTSK	PTGES	VDR	MET
PSAP	PPARD	POK1	GP81	CFD	NGO1	DHFR	ADH1C	PDQ2	IGR	BIRC7	MTHFD1	HMMT	ALDH2
NR1H3	MMP12	GLO1	EPHX2	RBP4	ADH5	TEK	F10	ERBB4	TGM3	FNTA	FABP3	MAP8B	ACP3
NR3C2	PNMT	WAS	MAPK1			SRC	ESR2	FGFR1	SHBG	PLA2G10	ZAP70	HPGDS	AKR1C3
TGFBF1	KDR	HSD17B1	TRAPPC3	GJA1	SERPINA1	LK1	PK3CG	PTPN11	POF	HMBG1	ITGAL	MMP8	HSD17B11
EGFR	CNTN1	SEC14L2	YARS1	CTNNA1	IGF1	SULT2B1	PPARA	IGF1R	PDE4D	NR1H2	KIF11	ESRRA	CDK2
CHEK1	HSPA8	NR1H4	CA2	FABP6	NR12	HDAC2	ITPA	MMP3	NR13	CDK6	HDAC8	THRA	SYK
SULT2A1	AKR1B10	GSTP1	CFB	JAK3	LCN2	GSK3B	EPH4	HSD11B1	PNP	OSR	PLK1	RORA	CRABP2
ALK	GSTA1	FABP5	HSD11B2	ELANE	GM2A								

C

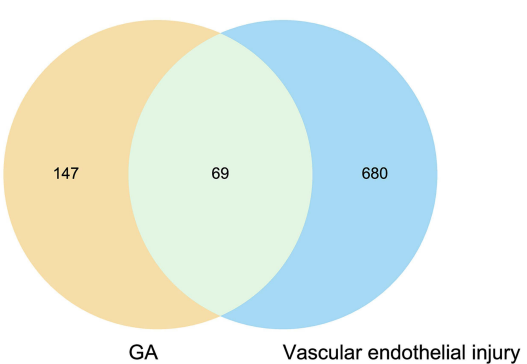
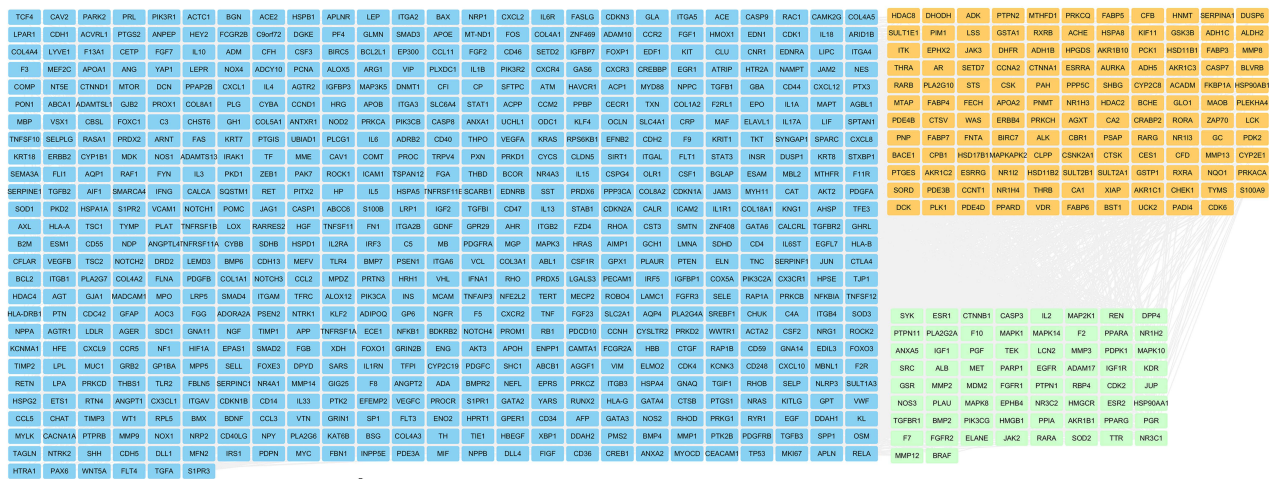


Figure 1 Target databases were applied to summarise the targets of 18β-glycyrretinic acid (GA) and vascular endothelial injury. (A) Summary of vascular endothelial injury targets using PubMed database and GeneCards databases; (B) Summary of GA using the drug target database; (C) The common vascular endothelial injury targets and GA targets were summarised by the Venn software.

A



Betweenness>769.8762878063
 Closeness>0.38604404168554
 Degree>20

B



C

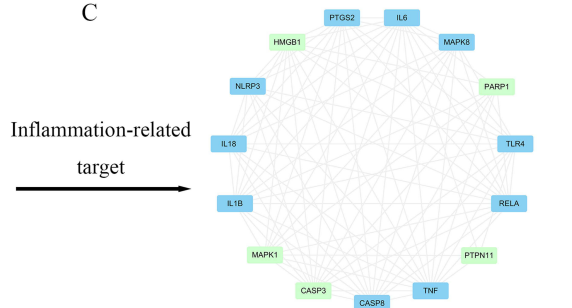


Figure 2 The protein-protein interaction (PPI) network and Cytoscape analyses were applied to screen the core targets of 18β-glycyrrhetic acid (GA) in alleviating vascular endothelial injury. **(A)** PPI network diagram after String database screening; **(B)** Diagram of core targets for further screening based on the Cytoscape database. Orange nodes represent GA targets, blue nodes represent vascular endothelial injury targets, and green nodes represent common targets of vascular endothelial injury and GA. **(C)** Screening of core targets related to inflammation.

and vascular endothelial injury targets were analysed by the Venn diagram (Figure 1C, Supplementary Table 1). To further reveal the core targets of GA and vascular endothelial injury, all targets were mapped into the STRING database and conducted PPI network. As shown in Figure 2A and B, blue nodes represent vascular endothelial injury targets, orange nodes represent GA targets, and green nodes represent common targets. The core targets of GA and vascular endothelial injury were further selected, including PARP1, HMGB1, IL-1β, IL-18, RelA (NF-κB p65), PTPN11 and MAPK1 (Figure 2C). In addition, this study also compared the GA docking scores with niraparib (FDA-approved PARP1 inhibitor) docking scores, the docking scores of niraparib and GA with PARP1 are 5.9030 and 5.4304, respectively, indicating that both niraparib and GA have the potential to bind with PARP1 (Figure 3 and Table 1). Collectively, these data suggest that GA alleviated *P. multocida*-induced vascular endothelial inflammatory injury, possibly by altering the expression of core targets such as PARP1, HMGB1, IL-1β, IL-18 and NF-κB p65.

Core Targets Participate in Inflammatory Disorders

To understand the putative functions of the core targets, they were mapped to the GO and KEGG databases for analyses. Among the enriched biological processes, some processes related to the regulation of inflammatory injury were mainly enriched, such as positive regulation of interleukin-8 production, cellular response to lipopolysaccharide (LPS), positive regulation of IκBα kinase/NF-κB signalling and inflammatory response (Figure 4A). According to the KEGG analysis, core targets involved in the top 40 signalling pathways were mainly involved in the PI3K-AKT signalling pathway, the MAPK signalling pathway, coronavirus disease-COVID-19 and the JAK-STAT signalling pathway (Figure 4B). Based on

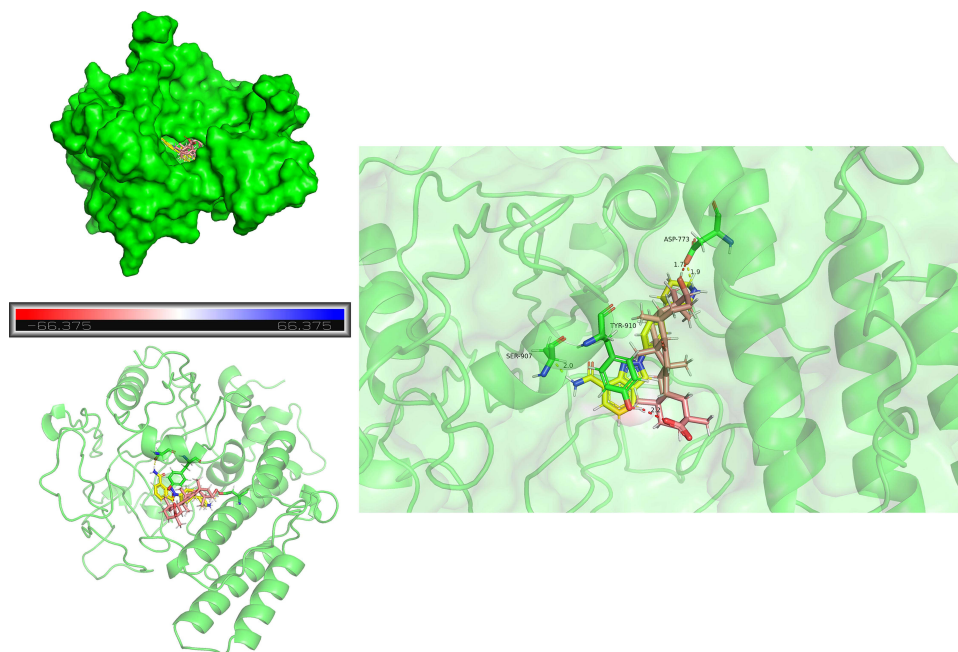


Figure 3 Molecular docking was applied to model the potential of 18 β -glycyrrhetic acid (GA) and niraparib to bind PARP1. Brown stick molecules represent GA, yellow stick molecules represent niraparib and green stick molecules represent amino acid residues of PARP1.

the results of the comprehensive GO and KEGG analysis, core targets of GA and vascular endothelial injury may participate in the inflammatory disorders of vascular endothelial cells.

Dose-Effect of GA on the Cytotoxicity of PIEC Cells

The CCK-8 assay was used to evaluate the dose-effect of GA on the cytotoxicity of PIEC cells. As presented in Figure 5A, below 40 $\mu\text{g}/\text{mL}$, GA was almost non-cytotoxic, with a cell viability > 80%. However, at concentrations of 80–160 $\mu\text{g}/\text{mL}$, GA exhibited significant cytotoxicity to PIEC cells ($p < 0.01$). Consequently, 10, 20 and 40 $\mu\text{g}/\text{mL}$ were used as the working concentrations of GA for the whole experiment.

GA Attenuates *P. multocida*-Induced Vascular Endothelial Inflammatory Injury

To evaluate whether GA can attenuate endothelial inflammatory injury, this study modelled vascular endothelial inflammatory injury using PIEC cells induced by *P. multocida*. The minimum inhibitory concentration (MIC) of GA against *P. multocida* was 1024 $\mu\text{g}/\text{mL}$. In addition, the western blot results showed that the expression levels of PARP1, p-NF- κB p65, HMGB1, IL-1 β and IL-18 were upregulated after *P. multocida* infection ($p < 0.01$), with a significant dose-dependent reversal of *P. multocida*-induced inflammatory protein (PARP1, p-NF- κB p65, HMGB1 and IL-1 β) expression by 10–40 $\mu\text{g}/\text{mL}$ of GA ($p < 0.01$) (Figure 5B and C); 10 $\mu\text{g}/\text{mL}$ ($p = 0.950$) and 20 $\mu\text{g}/\text{mL}$ ($p = 0.869$) concentrations of GA did not produce a significant effect on *P. multocida*-induced expression of IL-18. However, the concentration of 40 $\mu\text{g}/\text{mL}$ of GA significantly reduced the expression of IL-18. These findings indicate that *P. multocida* infection can cause an abnormal increase in inflammatory markers of vascular endothelial cells, whereas GA can effectively alleviate *P. multocida* infection-induced vascular endothelial inflammatory injury.

Table I The Docking Value of 18 β -Glycyrrhetic Acid (GA) and Niraparib with PARP1

Chemicals	Docking Score	Binding Site
GA	5.4304	ASP-773, TYR-910
Niraparib	5.9030	ASP-773, SER-907

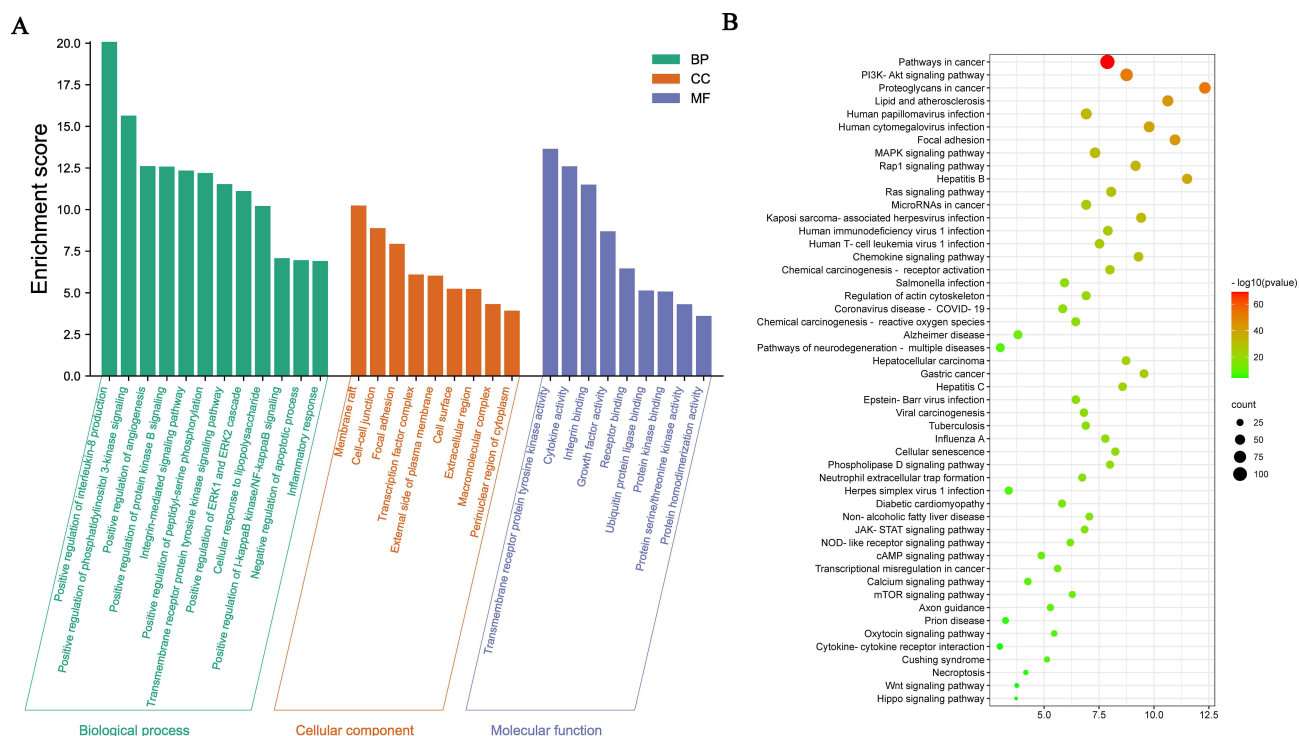


Figure 4 GO enrichment analysis and the Kyoto encyclopedia of genes and genomes (KEGG) analysis of the core targets of 18 β -glycyrrhetic acid (GA) in alleviating vascular endothelial inflammatory injury using the database for annotation, visualisation and integrated discovery (DAVID). (A) GO analysis of core targets; (B) KEGG analysis of core targets.

GA Attenuates *P. multocida*-Induced Vascular Endothelial Inflammatory Injury by Inhibiting the PARP1 Pathway

To gain insight into how GA alleviates the cellular inflammatory damage induced by *P. multocida* infection, a comprehensive analysis was performed in combination with PARP1 overexpression and interference assays, bacterial infection and GA treatment. As shown in Figure 6A and B, overexpressed PARP1 significantly promoted the expression of PARP1, p-NF- κ B p65, HMGB1, IL-1 β and IL-18 ($p < 0.01$). However, GA was able to alleviate inflammatory protein elevation induced by PARP1 overexpression in a concentration-dependent manner. Knockdown of PARP1 also down-regulated the expression of inflammatory proteins such as PARP1, p-NF- κ B p65, HMGB1, IL-1 β and IL-18 ($p < 0.01$) and inhibited the increase in inflammatory proteins induced by *P. multocida* (Figure 7A and B). The results showed that GA alleviates *P. multocida*-induced vascular endothelial inflammatory injury through the PARP1-HMGB1 or the PARP1- NF- κ B p65 signalling pathway.

Discussion

As one of the main pathogenic bacteria causing respiratory diseases in humans and animals, the study of the interaction between *P. multocida* and vascular endothelial cells plays an important role in exploring the pathogenesis of hemorrhagic pneumonia. The clinical treatment of *P. multocida* infections mainly occurs via the application of antimicrobials. However, antimicrobials do not solve the problem of inflammatory injury of vascular endothelial cells caused after *P. multocida* infection, and the massive and illegal use of antimicrobials is often accompanied by the occurrence of antimicrobial resistance.²⁷ Therefore, it is of great significance to explore the mechanism of vascular endothelial inflammatory injury induced by *P. multocida* infection and to search for new, effective drugs for adjuvant treatment or the control of inflammation in the disease to reduce the harm of *P. multocida* infection. In the present study, we used PIEC cells as a model and found that GA alleviates *P. multocida*-induced vascular endothelial inflammation by PARP1-mediated NF- κ B and HMGB1 signalling suppression (Figure 8), suggesting that GA and PARP1 are potential alternatives to antibiotics and candidate therapeutic targets for the adjuvant treatment of *P. multocida* infection, respectively.

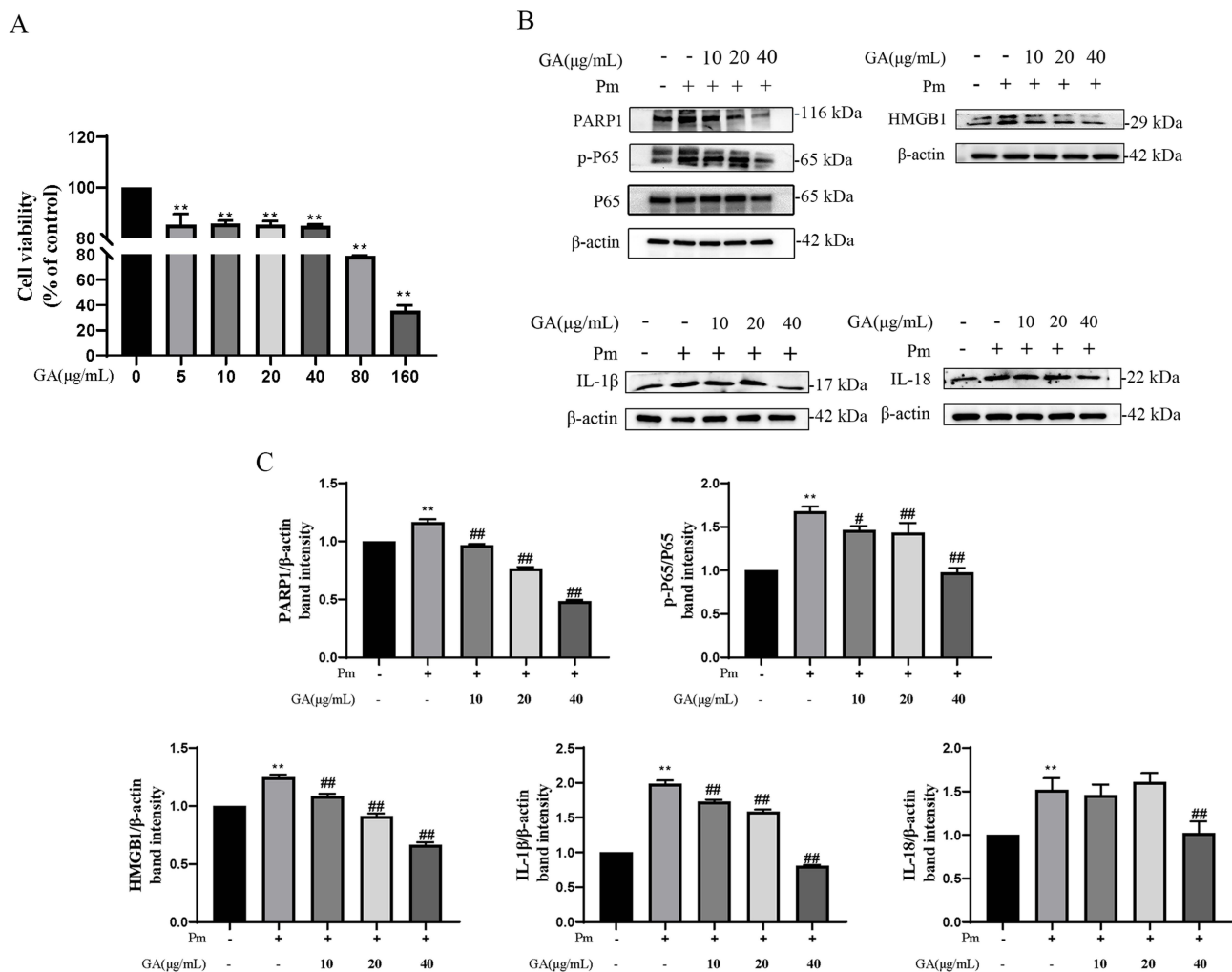


Figure 5 18β-glycyrrhetic acid (GA) attenuates vascular endothelial inflammatory injury induced by *P. multocida* infection in PIEC cells. **(A)** Cytotoxicity of GA on PIEC cells using the CCK-8 assay; **(B)** GA reduced the expression of inflammatory proteins induced by *P. multocida* infection in PIEC cells; **(C)** Protein density quantified using the ImageJ software. **represents the control group vs *P. multocida* infection group, $p < 0.01$; # and ## represent other groups vs *P. multocida* infection group, $p < 0.05$ and $p < 0.01$, respectively.

Network pharmacology is a new discipline to study the mechanism of drug-target-disease interactions in the context of larger biological networks.²⁸ To explore the possible target of GA in host cells that are responsible for the vascular endothelial inflammatory injury induced by *P. multocida* infection, network pharmacology analysis was conducted. Some inflammation-related core targets of GA and vascular endothelial injury were enriched by PPI network and Cytoscape analysis,^{29,30} such as PARP1, HMGB1, IL-1β, IL-18, NF-κB p65, indicating that inflammatory pathways play an important role in GA alleviating *P. multocida*-induced vascular endothelial inflammatory injury. Moreover, GO analysis showed that core targets are mainly enriched in biological functions related to the regulation of inflammatory injury, such as positive regulation of interleukin-8 production, cellular response to LPS, positive regulation of IκBα kinase/NF-κB signalling and inflammatory response. Based on the results of the KEGG enrichment analysis, GA involves multiple signalling pathways in the treatment of *P. multocida* infection, mainly including the PI3K-AKT signalling pathway, the MAPK signalling pathway, coronavirus disease-COVID-19 and the JAK-STAT signalling pathway. In addition, studies have shown that the NF-κB/MAPK signalling pathway is involved in the regulation of LPS-induced inflammatory responses in IPEC-J2 cells.³¹ According to another study, HMGB1 can enhance the inflammatory response by activating the p38 MAPK signalling pathway.^{32,33} Meanwhile, PARP1 could serve as a regulator of inflammatory diseases through promoting NF-κB activation and HMGB1 secretion.^{13,14} The network pharmacology results shown herein revealed that the activation of PARP1 might contribute to the activation of NF-κB and HMGB1, which may finally be beneficial for vascular endothelial inflammatory injury after *P. multocida* infection.

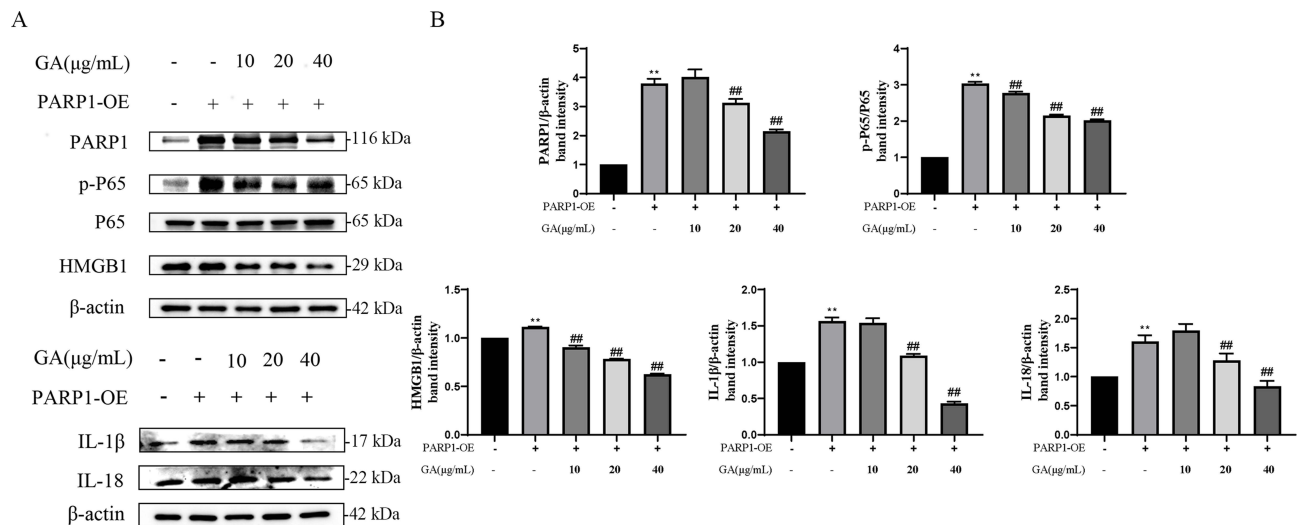


Figure 6 18β-glycyrrhetic acid (GA) alleviates overexpressed PARP1-induced vascular endothelial inflammatory injury in PIEC cells. **(A)** Pre-treatment with GA could alleviate the overexpressed PARP1-induced expression of inflammation-related protein, such as PARP1, HMGB1, p-P65, IL-18 and IL-1β; **(B)** Protein density quantified using the ImageJ software. PARP1-OE represents overexpressed PARP1. **Represents the control group vs the PARP1-OE group, $p < 0.01$; ###Represents other groups vs PARP1-OE group, $p < 0.01$.

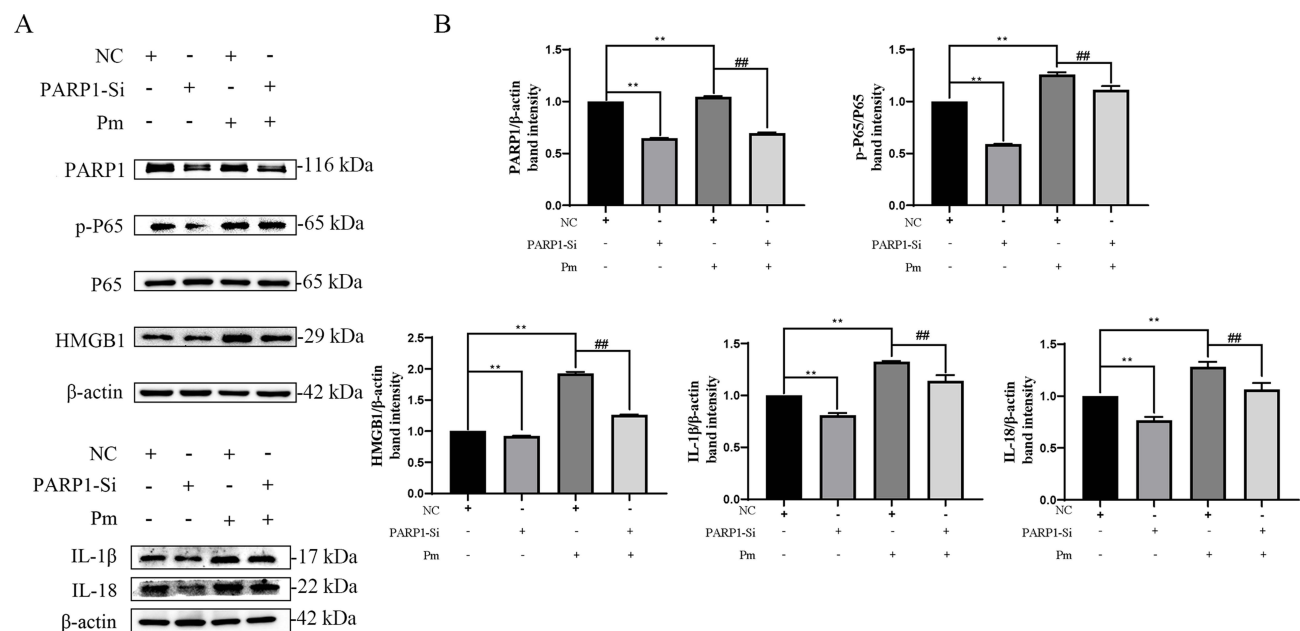


Figure 7 Knockdown of PARP1 decreased vascular endothelial inflammatory injury induced by *P. multocida* infection in PIEC cells. **(A)** Knockdown of PARP1 decreased *P. multocida* infection-induced expression of inflammation-related protein, such as PARP1, HMGB1, p-P65, IL-18 and IL-1β; **(B)** Protein density quantified using the ImageJ software. PARP1-Si represents Knockdown of PARP1. NC represents negative control siRNA. ###Represents NC group+ *P. multocida* infection group, $p < 0.01$; **Represents other groups vs NC group, $p < 0.01$.

The GA can be an anti-inflammatory drug candidate for the treatment of inflammatory responses evoked by various external stimuli. In a previous study, it inhibited *Staphylococcus aureus*-induced RAW264.7 cell inflammation by inhibiting the activation of NF-κB and the expression of HMGB1¹⁹ and showed a protective effect on *Helicobacter pylori*-infected gastric mucosa through alleviating the expression levels of inflammation-related cytokines (IL-1β, TNF-α).²⁰ Further, GA suppressed allergic airway inflammation through NF-κB and Nrf2/HO-1 signalling pathways in an ovalbumin-induced asthma mouse model.³⁴ In LPS- or TNF-α-induced inflammatory cell models and collagen-induced arthritis animal models, GA exerts its anti-inflammatory activity through the MAPK/NF-κB signalling pathway.³⁵ The above studies demonstrate

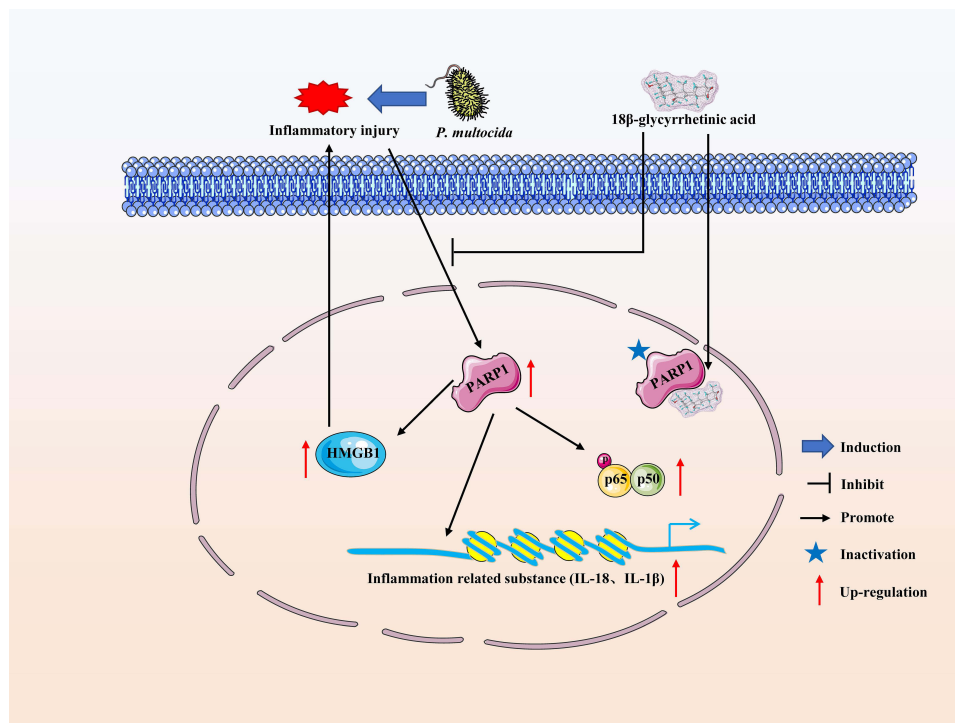


Figure 8 18 β -glycyrrhethinic acid (GA) alleviates *P. multocida*-induced vascular endothelial inflammation by PARP1-mediated NF- κ B and HMGB1 signalling suppression in PIEC cells. *P. multocida*-infected PIEC cells could significantly activate the PARP1-mediated NF- κ B and HMGB1 signalling pathways and induce the PARP1-mediated expression of inflammatory-related substances, such as IL-1 β and IL-18. On the one hand, GA may inhibit the activity of PARP1 by targeting it, on the other hand, it may inhibit the NF- κ B and HMGB1 signalling pathways by reducing the expression of PARP1, thus inhibiting *P. multocida* infection-induced vascular endothelial inflammatory injury in PIEC cells.

that GA presents great anti-inflammatory activity *in vivo* or *in vitro* by inhibiting pathways such as NF- κ B and HMGB1. This study found that GA could inhibit the NF- κ B and HMGB1 pathways to alleviate the vascular endothelial inflammatory injury induced by *P. multocida* infection. In addition, in this study, GA potentially targeted PARP1 by network pharmacology and molecular docking technology, which in turn exerted the inhibition of NF- κ B and HMGB1 pathway activation.

The PARP1 is involved in the regulation of inflammation in bacterial infections. In LPS-induced mouse septic shock and *S. typhimurium*-induced mouse colitis models, the absence of PARP1 decreased NF- κ B-mediated inflammatory disorders.^{16,36} Moreover, PARP1 mediates LPS-induced HMGB1 secretion from macrophages, and PARP1 activity-dependent HMGB1 translocation from the nucleus to the cytoplasm can promote the activation of cellular inflammatory pathways.¹⁴ However, loss of PARP1 activity impedes the cytosolic translocation of HMGB1, which can prevent the HMGB1-mediated TLR4- NF- κ B signalling pathway to attenuate LPS-induced myocardial inflammation.³⁷ In the present study, either PARP1 overexpression or PARP1 deletion demonstrated the ability of PARP1 to promote the expression of NF- κ B and HMGB1, which in turn were involved in the progression of vascular inflammatory injury induced by *P. multocida* infection. In addition, the molecular docking score is greater than 4,³⁸ implying that PARP1 might be a key target of GA. And GA at lower antimicrobial concentrations (10 ~ 40 μ g/mL) on the inflammatory response of endothelial cells induced by *P. multocida* infection. Thus, we speculate that GA may play a role in alleviating vascular endothelial inflammatory injury induced by *P. multocida* infection by targeting PARP1 and inhibiting PARP1 activity. The above studies demonstrate that GA and PARP1 could serve as a novel antibiotic alternative and therapeutic target for the prevention and treatment of vascular endothelial inflammatory injury induced by *P. multocida* infection, respectively.

This study has, however, some limitations. First, some unconfirmed and unrecorded targets of the target database may not be enriched in the present study, implying that the target database of network pharmacology needs to be further improved. Second, the molecular docking results showed that PARP1 may have the potential to bind with GA, but its specific binding situation awaits further investigation. Third, the results of the present study showed that PARP1 could regulate the protein expression of NF- κ B and HMGB1, but whether GA inhibits inflammation by targeting PARP1 to regulate the nuclear

translocation of NF- κ B and HMGB1 during *P. multocida* infection awaits further exploration. Fourth, pharmacodynamic and animal therapeutic trials are needed to further verify the potential of GA as an alternative to antibiotics in the treatment of vascular endothelial inflammatory injury caused by *P. multocida* infection.

Conclusion

Collectively, through the mining of network pharmacology studies and the verification of in vitro experiments, this study proves the powerful potential of network pharmacology in disease and drug target screening and that PARP1 is a potential target for GA for the treatment of vascular endothelial inflammatory injury caused by *P. multocida* infection. Further, PARP1-mediated NF- κ B and HMGB1 signalling suppression may be a critical signalling pathway for treating vascular endothelial inflammatory injury caused by *P. multocida* infection.

Ethics Statement

Part of the target data for this manuscript was obtained from a public database, such as GeneCards. The ethics committee of Wuhan Polytechnic University has reviewed the data in this manuscript, which does not violate relevant ethical issues and agrees to publish it.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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