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ORIGINAL RESEARCH

Antiviral Activity of Oridonin Against Herpes Simplex Virus Type 1

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Purpose: In search of new potent treatment of herpes simplex keratitis (HSK), inhibitory effect of oridonin (Ori) on herpes simplex virus type 1 (HSV-1) was validated by experiments.

Methods: For evaluating inhibitory effect of oridonin on herpes simplex virus type 1, a series of in-vivo and in-vitro studies were carried out. Mouse HSV-1 infection model was used in the in-vivo experiments. Experimental mice were classified in five different groups: Mock (mock-infected), HSV-1+ DMSO, HSV-1+ Ori, HSV-1+ ACV, combined Ori and ACV+HSV-1. Corneas of Mock, HSV-1+ DMSO, HSV-1+ Ori group were sent for mRNA-sequencing after 3 days post infection (dpi). The expression of virus and host-related genes was evaluated by quantitative real-time polymerase chain reaction (qPCR). Vero cells HSV-1 infection models were used in the in-vitro experiments.

Results: The application of ACV, Oridonin alone or a combination of both could alleviate HSV-1 severity and inhibit HSV-1 virus replication in C57BL/6 mice models. qPCR showed that compared with mock group, the expression of *interleukin-6 (il-6), interleukin-1α (il-1α)*, and *Tumor-necrosis factor-alpha (tnf-α)* was up-regulated in DMSO+HSV-1 group and suppressed in other three group. Moreover, the expression of nod-like receptor protein *(nlrp3), caspase 1 and interleukin-1β* (*il-1β*) were depressed in the oridonin-treated group. Oridonin significantly inhibits HSV-1 replication, HSV-1 related gene expression, and the production of progeny HSV-1 viruses in vitro. Besides, oridonin affect the replication phase but not HSV-1 entry or penetration and cannot inactivate HSV-1.

Conclusion: Oridonin alleviates herpes simplex keratitis infection in mouse, which may be attributed to inhibition of the NLRP3-inflammasome-IL-1β pathway. Our study illustrates that Oridonin has potential promise for application in treating HSK and other diseases caused by HSV-1 infection.

Keywords: oridonin, HSV-1, herpes simplex keratitis, herb, *nlrp3*, inflammasome

Introduction

Human health is still being menaced by virus infection.[1] Herpes simplex virus type 1 (HSV-1) infection is a common cause of other serious diseases in addition to HSK, including oral mucosal lesions, encephalitis, and meningitis.[2–4] Herpes simplex keratitis (HSK) is a recurrent inflammation in cornea induced by HSV-1 infection, which can seriously threaten vision and has become one of the most common infectious diseases causing blindness with a high incidence worldwide. In 2016, a preliminary estimated 1.7 million people had HSK, and over 200,000 people among them acquired unilateral vision loss caused by HSK.[5] Repeated episodes of stromal keratitis have been established to often lead to permanent scarring and visual impairment, which requires corneal transplantation.[6,7] No vaccine has currently been licensed to prevent HSV-1 infection,[8] although several drugs, such as acyclovir (ACV), ganciclovir, famciclovir, valaciclovir, foscarnet, and cidofovir, are applied in the treatment of HSK.[9] A handful of drugs such as topical formulations of idoxuridine, trifluridine and vidarabine are also approved for curing recurrent HSK.[10] Nevertheless, long-term usage of ACV can cause side effects.[11] Given that resistance associated with ACV has gained significant momentum in recent years,[12] long-term treatment of HSK has become challenging. Therefore, exploring new therapeutic agents is still essential to controlling HSV-1 infection.

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Rubescens has been used for treating tonsillitis in traditional Chinese medicine for centuries.[13] *Rubescens*, available in tablet and drop/pill forms, can relieve pain[14] or enhance the chemotherapeutic drug efficacy.[15] Ori is a natural diterpenoid extracted from *Rabdosia rubescens*.[16,17] Ori can be applied as an adjuvant therapy with other chemotherapy drugs to cure cancers, such as leukemia,[18] gastric cancer,[19] and breast cancer.[20] In recent years, many studies have found that Ori has other health-promoting effects, including a protective effect on acute liver injury,[21] anti ROS,[22] anti microbiotics,[23] suppressing depressive behaviors,[24] and even neuroprotection against Alzheimer's Disease.[25,26] It was demonstrated that Ori might inhibit enterovirus D68 production and coronaviral replication in vitro.[27,28] Little is known about the inhibitory effect of Ori against HSK yet.

Ori was used to mitigate HSK in mouse model and inhibit HSV-1 virus replication in vitro in this study. We used RNA-sequencing to further probe genes and pathways included in the treatment of HSK through Ori injection in order to build a theoretical foundation for developing Ori as a potential approach in HSK therapy.

Materials and Methods

Agent

Ori and ACV were purchased from Med Chem Express (Shanghai, China). DMEM medium, FBS, trypsin, penicillin G (10,000 U/mL) and streptomycin (10 mg/mL) were purchased from Gibco (Carlsbad, USA). Fetal bovine serum (FBS) was obtained from ExCell Bio (Hongkong, China).

Mice

This experiment used healthy male C57BL/6 mice, 6–8 weeks old, bought from Weitong Lihua Limited Company (Beijing, China). Mice were exposed to a light/dark cycle for 12h in an indoor environment (22±2°C) at the Shandong Eye Institute with free access to food and water. All laboratory animals were fed and treated according to the laboratory animal protection provisions. The Ethics Committee approved the Shandong Eye Institute animal study protocol (protocol code 20210805–01).

Cell Culture

African green monkey kidney (Vero) cells were obtained from Shandong Eye Institute. Vero cells were cultured in DMEM/F12 containing 10% FBS, 100 U/mL of penicillin and 100 mg/mL of streptomycin. Cells were cultured in dishes at 37°C and 5% CO_2. When cell density reached 80% to 90% confluence, cells were used for subculture.

Virus

The H129-G4 HSV-1 strain virus containing a CMV promoter marked with EGFP was bought from Shanghai Genechem Co. (Shanghai, China). Vero cells were infected with H129-G4 and HSV-1-McKrae viruses titered by the plaque assay. Briefly, Vero cell monolayers in a 24-well plate were infected with the virus after serial dilution (10-fold) when the cell density reached 90%. The plate was shaken slightly once every 15 min per hour. After 4–6 h, the excess HSV-1 virus was removed by washing. DMEM/F12 containing 2% FBS and 1.2% agarose was added to cover the cells (0.6 mL/well), and the plate was placed in a cell incubator for 4–6 days. The agarose layer was removed, and 4% paraformaldehyde was added to fix the cells for one day (0.6mL/well). 0.3mL of a 0.2% crystal violet solution and PBS mixture was added for staining. After 24 h, plates were washed with water and the PFU was counted.[29] The viral infection titer was expressed as PFU/mL.

qPCR

Nucleospin RNA Kit (Takara, Dalian, China) was used to extract the total RNA from samples. cDNA was synthesized using Primescript RT Master Mix (Transgen, Beijing, China) obeying the manufacturer's guidance. qPCR was launched using SYBR Premix Ex Taq II (Vazyme, Nanjing, China) and Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA) to measure HSV-1 virus DNA. The cycle condition was 95°C for 30s, followed by 40 two-step cycles (95°C for 5s, 60°C for 30s). The primers for the genes RPL5, HSV-1 ICP0, TK, VP16, *mrpl5, il-6, il-1α, tnf-α, nlrp3, caspase 1 and il-1β* are provided in Table 1 and the relative gene expression was determined based on deactivation with the housekeeping gene as described previously.[30]

Table 1 Primers Used for qPCR

No.	Gene	Forward Primer	Reverse Primer
1.	RPL5	GGAAGCACATCATGGGTCAGA	TACGCATCTTCATCTTCCTCCATT
2.	ICP0	GGCCCCCTTGTCAACAGA	GGGAGTCGCTGATCACTATGG
3.	VP16	GCGGGGCCGGGATTTACC	CTCGAAGTCGGCCATATCCA
4.	TK	AAGGTCGGCGGGATGAG	CGGCCGCGCGATAC
5.	*il1b*	CTTTCCCGTGGACCTTCCA	CTCGGAGCCTGTAGTGCAGTT
6.	*tnf-a*	ACAAGGCTGCCCCGACTAC	TGGGCTCATACCAGGGTTTG
7.	*il6*	ACCACTCCCAACAGACCTGTCT	CAGATTGTTTTCTGCAAGTGCAT
8.	*il1a*	GATGAAGCTCGTCAGGCAGAA	CCTCCCGACGAGTAGGCATA
9.	*casp1*	CTGGGACCCTCAAGTTTTGC	CCCTCGGAGAAAGATGTTGAAA
10.	*m-rpl5*	CCGCAGGCTTCTGAATAGGT	CCAGTTGTAGTTCGGGCAAGA
11.	*nlrp3*	CTGCGGACTGTCCCATCAAT	AGGTTGCAGAGCAGGTGCTT

HSK Model in Mice and Treatment

All animal assays were consented by the Animal Protection and Use Committee of Shandong Eye Institute affiliated to Shandong First Medical University. All animals were weighed and divided into five groups at random (n = 6). Each mouse was anesthetized with an intraperitoneal injection of 0.6% pentobarbital (50mg/kg). As mentioned previously, the cornea epithelium of mouse in each group was scraped off with a 25 g needle under a surgical microscope.[31] The eyes were then treated with 5ul HSV-1 (McKrae strain) suspension containing 10^6PFU/mL. In Mock group mice, corneas were scratching and then treated with DMEM/F12 containing 2% FBS solution. Began at −1 day post infection, the mice were treated daily by peritoneal injection with 6mg/kg DMSO, 50mg/kg ACV, 20mg/kg Ori, 25mg/kg ACV and 10mg/kg Ori. ACV is a nucleoside analog used to treat varicella-zoster virus and HSV infections.[32] At 3 dpi, the progress of clinical corneal lesions in mice was examined by a slit-lamp biological microscope (Topcon, Nagoya, Japan). Then, the clinical severity of keratitis according to the degree of corneal inflammation in each mouse was recorded and scored.[33] The scoring criteria was as following: 0, clear cornea; +1, mild corneal opacity; +2, moderate corneal opacity or scar; +3, severe corneal opacity, but iris visible; +4, corneal opacity and ulceration; +5, necrotizing stromal keratitis.

In a separate experiment, tear samples (n = 6) were harvested by cotton swabs on 3 dpi, as previously mentioned.[34] All swabs were immersed in 600μL of DMEM with 2% FBS and frozen at −80°C. The TCID50 assay was used to measure the HSV-1 titers in tear samples. Briefly, tear sample was 10-fold serially diluted and titered on Vero cell monolayers. Virus titer was expressed as TCID50/mL according to the Spearman–Karber method.[35] The mice were sacrificed at 3 dpi, corneal samples were harvested, and tissue homogenates were obtained. RNA was extracted and reversed into cDNA as mentioned above. PCR assays were carried out to quantify the amount of TK, VP16, and ICP0 and inflammatory indexes such as IL6, IL1α, TNFα in the samples.

Histological Analysis

The cornea samples (n = 6) were fixed in paraffin oil for 24 h in an indoor environment. Then tissues were dehydrated in ethanol and infiltrated with 100% xylene. The samples were embedded in paraffin and cut into 5 μm sections after that. The cornea sections dyed with hematoxylin and eosin (HE) were examined using a conventional microscope (Nikon, Japan).

Confocal Microscope

The mice were sacrificed by cervical dislocation at 3 dpi, then the corneas of each group (n = 6) were harvested for immunofluorescence staining of frozen sections. Briefly, slices were fixed with 4% paraformaldehyde, permeabilized

with 0.3% Triton X-100, blocked with 10% donkey serum, incubated with HSV-1 ICP0 antibody (rabbit polyclonal from Abcam, Cat. No. ab9533) (1:200). After washing with Wash buffer, a Donkey Anti-Rabbit IgG H&L (Alexa Fluor 488, Abcam, Cat. No. ab150073) (1:1000) was added for 1 hour. The staining slices were examined by laser scanning confocal microscopy (Carl Zeiss Meditec AG, Jena, Germany). In cell model, GFP is used to detect the recombinant virus. The excitation wavelength of GFP was 488 nm, and that of DAPI was 408 nm.

Cell Viability

Vero cells (10^4 cells/well) were seeded in 96 well plates and treated with different concentrations of Ori 24 h later. After 48 h, the survival rate of cells was examined using the LDH Assay Kit (Beyotime Biotechnology, Beijing) following the manufacturer's guidance.[36] We used linear regression method to determine the 50% cytotoxicity concentration (CC50).

In vitro Experiment

For immediate observation, HSV-1 marked with recombinant EGFP (G4 strain) was used in in-vitro assay. The HSV-1-G4 strains were used for infecting Vero cells, respectively, with different concentrations of Ori and Hoechst 33258 staining before photography. Fluorescence was used to measure the cell infection at 24hpi and 48hpi. Then Vero cells (1.5×10^5/ well) were inoculated in 24-well plates and incubated overnight. HSV-1 (McKrae strain) (1.5×10^4PFU/ well) at a multiplicity of infection (MOI)=0.1 was added and cells were treated with 6μM Ori, 1μM ACV, or a mixture of 6μM Ori and 1μM ACV. A control group (0.1%DMSO) was established. After three freeze-thaw cycles, the virus titers were determined by plaque assay at 24 hpi (hour post infection). In another experiment, Vero cells (2.0×10^6 /well) were incubated in 24-well plates overnight. The designated cultured cells were infected with HSV-1 (McKrae virus strain) at MOI = 5.0. For Mock group, a 2% FBS in DMEM/F12 solution was used. Samples were collected at 9 hpi. Then, RNA in samples was extracted, and qPCR was carried out by the above-mentioned method. Viral inactivation assay, anti-penetration assay, and anti-attachment assay were launched, then the pretreatment, posttreatment, and coaddition assays were performed using 6 μM Ori within different stages of HSV-1 infection in Vero cells.[37] Samples were collected, and RNA was extracted. The qPCR experiment was performed obeying the method described above.

Transcriptome RNA Sequencing Analysis

To determine the pathway by which Ori affects HSK in the mice model, mRNA sequencing was performed. Corneas of Mock, HSV-1+ DMSO, HSV-1+ Ori group were collected for mRNA-sequencing after 3 dpi. About 100 ng of RNA in each group was sonicated into 300- to 400-bp fragments using a bioruptor (Diagenode, Belgium), and sequencing libraries were prepared using a VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (NR603–02, Vazyme, Nanjing, China) following the producer's guidance. The RNA libraries were sequenced on an Illumina HiSeq2500 sequencer using a HiSeq SR Cluster Kit V4 (GD-401-4001, Illumina, San Diego, USA) and a HiSeq SBS Kit V4 50 cycle kit (FC-401-4002, Illumina, San Diego, USA). We used the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) to evaluating RNA purity and quantification. Then the libraries were created using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, USA). The clean reads were mapped to the GRCm38 mouse genome with HISAT2.[38] The fragments per kilo bases per million fragments (FPKM) of each gene was calculated using Cufflinks with the RefSeq mm9 reference transcriptome,[39,40] and the read counts of each gene were obtained by HTSeqcount.[41] Then, each sample's expression values were summed to detect significant differences in gene transcript expression between groups. Differentially expressed genes (DEGs) were determined using the DESeq (2012) R package with P-value < 0.01 and fold change > 2 or fold change < 0.5.[42] GO enrichment analysis of DEGs was performed using R based on the hypergeometric distribution.

Statistical Analysis

Statistical analyzes were performed using GraphPad Prism 9 software (GraphPad Prism, San Diego, CA, USA). All in vivo and in vitro experiment results are represented as the mean±SD from at least three independent experiments. Student's *t* test or one-way analysis of variance (ANOVA) were used for statistical analysis. The difference was considered statistically significant when P-value <0.05.

Results

Oridonin Treatment Alleviates HSK Severity in Mice Models

Slit-lamp examination was used to evaluate symptoms of HSK in mice models, and the scores were recorded. Ori could alleviate the symptoms of HSK in HSV-1+Ori group compared with that in HSV-1+DMSO group (Figure 1A). It was indicated that the amount of corneas scored ≤ 2 in the HSV-1+Ori group (10/12) was larger than that in the HSV-1 +DMSO group (0/12). We also found that the alleviating effect induced by the combination of Ori and ACV was significantly greater than in both groups alone ($p < 0.05$), while the number of cornea scored ≤ 1 in HSV-1+ACV+Ori group was 10/12 (Figure 1B). We used qPCR to evaluate the amount of inflammatory factors in samples. It was showed that the relative expressions of inflammatory factors in the HSV-1+Ori group were significantly lower than in the HSV-1 +DMSO group (> 30-fold decrease for *il-6*, > 2-fold decrease for *il-1α* and *tnf-α*, $p < 0.001$) (Figure 1C). As previous study reported, both IL-1α and IL-6 increase at 2 dpi, peaking at 10 dpi, and diminished over the next few days after

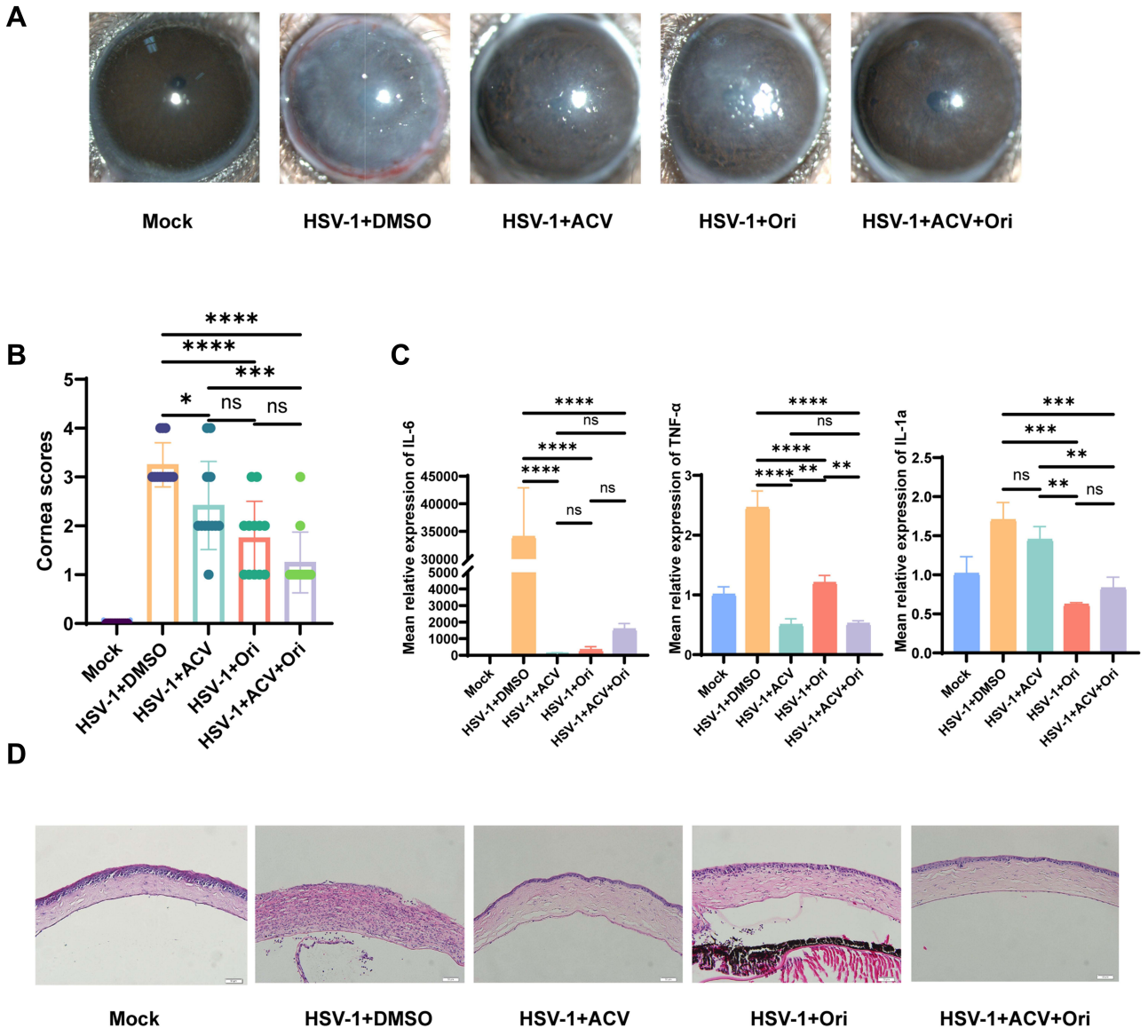

Figure 1 Oridonin relieves HSK severity in mice models. (**A**) Cornea opacity and neovascularization changes were monitored after application with DMSO, Ori, ACV, combined Ori and ACV at 3dpi. (**B**) Corneal clinical scores (n=12 per time point) were calculated. (**C**) expression of *il-1a, il-6* and *tnf-α* (n=6) were measured by qPCR at 3dpi. *mrpl5* was used as the reference gene. (**D**) Histological evaluation. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns means no significance, compared with the control group with PBS and DMSO injection). P values were calculated with one-way ANOVA.

HSV-1 infection.[43] Our findings suggest that Ori reduced the inflammation in HSK of mouse models. Pathological sections embedded in paraffin showed that corneal edema and inflammatory cells in the corneal stroma were reduced in the HSV-1+Ori group compared with the HSV-1+DMSO group (Figure 1D).

Oridonin Inhibits HSV-1 Replication in Mouse Models

In animal experiments, we found that Ori reduced HSV-1 titers in tears of mouse model at 3 dpi. The HSV-1 titers in tears of HSV-1+Ori group were more than 10-fold decreased than that of HSV-1+DMSO group (Figure 2A). Confocal microscope results showed that the expression of HSV-1 ICP0 was decreased in the HSV-1+Ori group and the HSV-1 +ACV+Ori group (Figure 2B). qPCR was performed on corneal tissues of mice sacrificed at 3 dpi, and the results showed that in HSV-1+Ori group and the combination group, the relative expression of TK, ICP0, and VP16 RNA, which represent the HSV-1 virus replication, was significantly reduced (> 2 fold, $p < 0.01$) than that of HSV-1+DMSO group (Figure 2C).

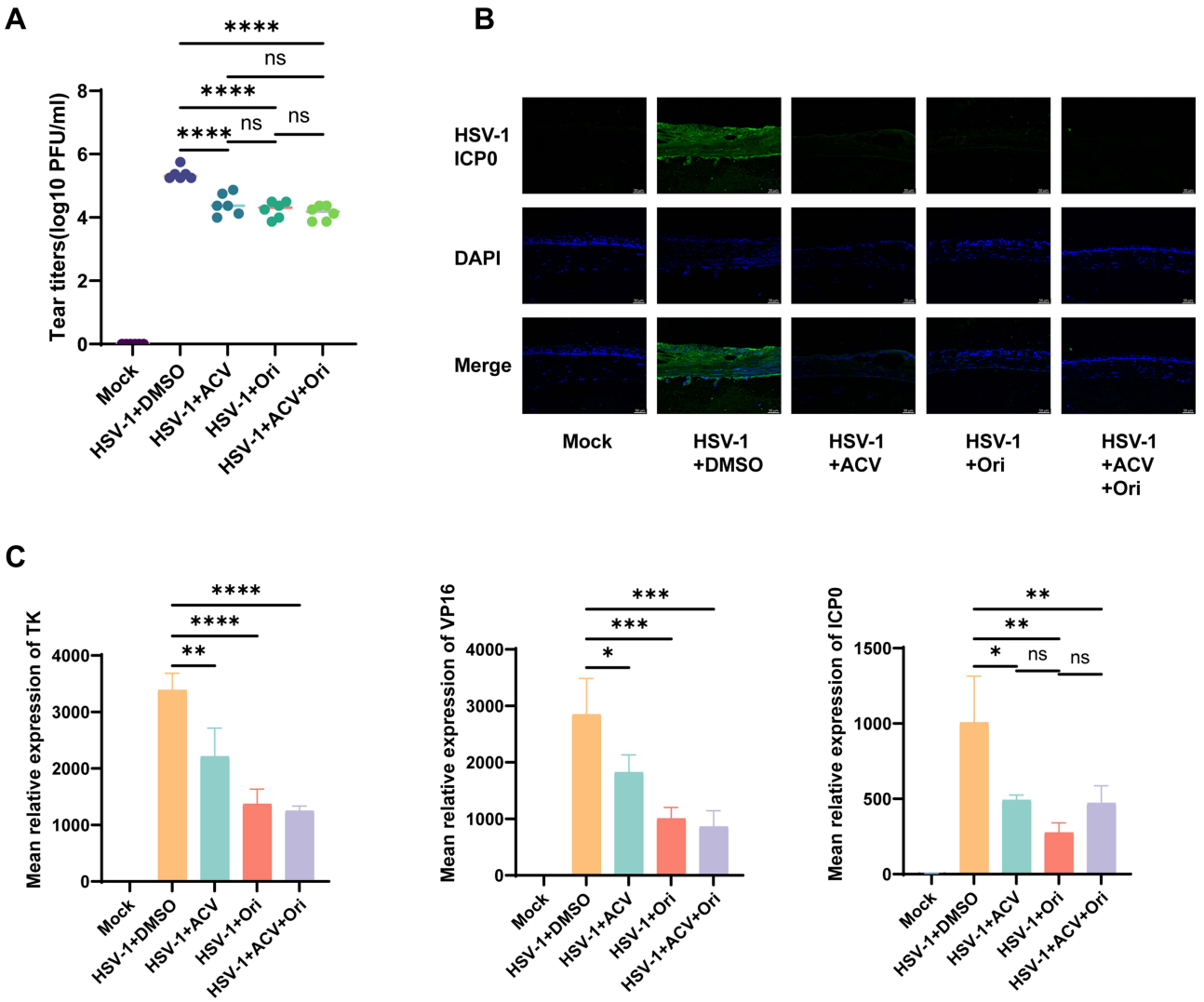

Figure 2 Ori inhibits HSV-1 virus replication in mouse models. (**A**) Tear titers (PFU/mL) obtained by TCID50 method at 3 dpi (n=6). (**B**) Representative immunostaining of corneal DAPI, HSV-1 ICP0 at 3dpi. (**C**) relative expression of TK, VP16, and ICP0 in cornea samples (n=6) were measured by qPCR. *mrpl5* was used as the reference gene. (*P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001, ns means no significance, compared with the mock group with 0.1%DMSO peritoneal injection). P values were calculated with one-way ANOVA.

Oridonin Inhibits HSV-1 Replication in vitro in the Post-Infection Stage

Before evaluating the inhibiting effect on HSV-1, the cytotoxicity of Ori was determined in Vero cells using the LDH Assay after 48 h of incubation. The results of the cell viability experiment showed that Ori did not exert obvious cytotoxicity at concentrations lower than 10 μM (Figure 3A). To validate Ori's efficiency, we used an EGFP-tagged HSV-1 strain infecting Vero cells with different concentrations of Ori. For control experiments, a 0.1% DMSO solution was used. The results showed that the 6 μM and 8 μM of Ori significantly inhibited HSV-1 replication in a concentration-dependent manner (Figures 3B and C). In Vero cell models, 6 μM Ori treatment showed a prominent inhibitory effect on HSV-1 replication by plaque assays (Figures 3D). The HSV-1 titers were calculated, and the titers in HSV-1+Ori group were significantly reduced (> 4-fold, $p < 0.0001$) than that in HSV-1+DMSO group. Moreover, Ori enhances antiviral activity with ACV against HSV-1 Infection (Figure 3E). qPCR analysis showed that the relative expression of TK, VP16 and ICP0 was significantly reduced ($p < 0.05$), and the inhibition effect of 6μM Ori on HSV-1 was similar to that of 1μM ACV (Figure 3F).

HSV-1 replicates and assembles inside the cell to release after forming a bond and fusion with the cell membrane. To further determine in which stage of the HSV-1 life cycle Ori acted, we performed several assays according to the protocol schematically shown in Figures 4A and B. Ori treatment did not affect HSV-1 attachment, entry stages or viral activity (Figure 4C-E). In contrast, Ori treatment efficiently impaired intracellular HSV-1 mRNA production with a more than 5-fold decreased expression of *VP16* at the post-infection stage (Figure 4F). However, Ori pretreatment of cells or coadministration with virus had no effect on HSV-1 replication (Figure 4F). These results show that Ori suppresses HSV-1 at replication stage in vitro.

RNA-Sequencing Identified the GO Terms and Signaling Pathways Regulated by Oridonin

We used RNA-sequencing (RNA-seq) in HSV-1 Mock group (DMEM+2%FBS) and HSK treated with 0.1%DMSO or Ori group to outline their gene expression patterns. Compared to the HSV-1+DMSO group, the HSV-1+Ori group significantly altered the expression of 413 genes (upregulated and downregulated) (Figure 5A). In the two-dimensional

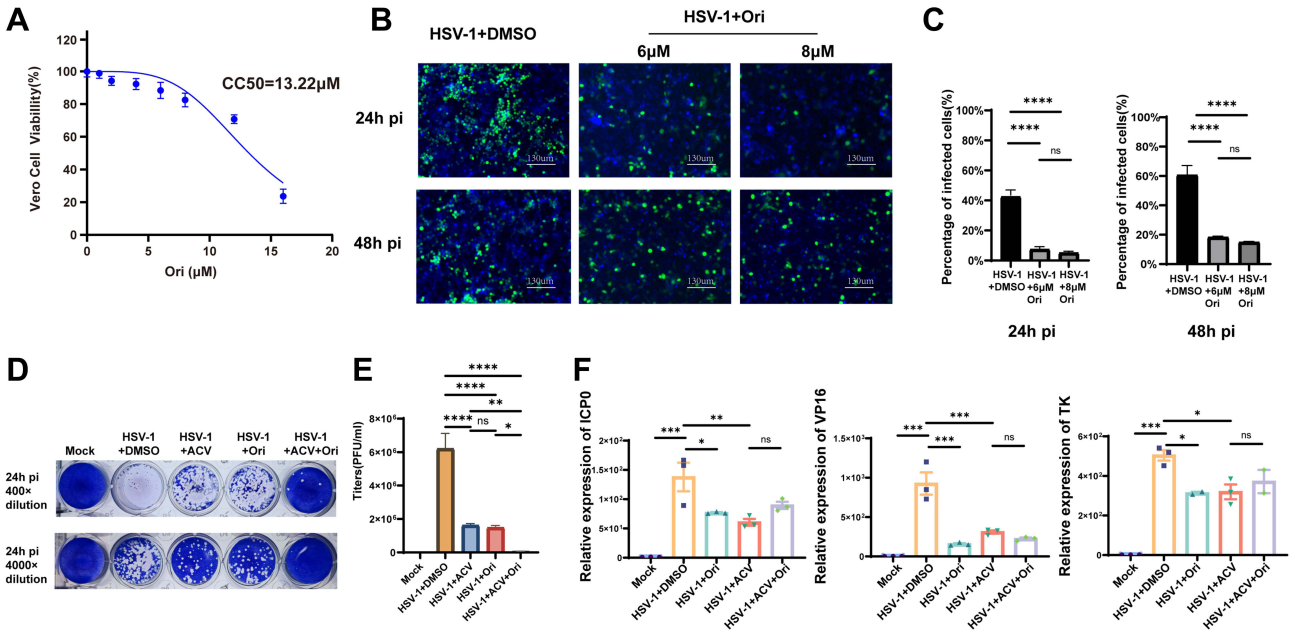

Figure 3 Ori disrupts on HSV-1 virus replication in vitro. (**A**) Cell viability of Ori in Vero cells. (**B**) Vero cells were infected with HSV-1-EGFP (MOI=1) for 2 h and then treated with various concentrations of Ori for 24h and 48h. For control experiments, a 0.1% DMSO solution was used. (**C**) The ratio of viral infection was quantified by EGFP fluorescence (n=6). (**D**) Plaque assays were performed and HSV-1 virus titers of each group (**E**) were calculated (n=6). (**F**) qPCR analysis of HSV-1 viral gene expression. Total RNA was isolated and subjected to qPCR analysis with the primers specific to HSV-1 TK, VP16, and ICP0 (n=3). Relative RNA expression was normalized to RPL5 in each sample. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns means no significance). P values were calculated with one-way ANOVA.

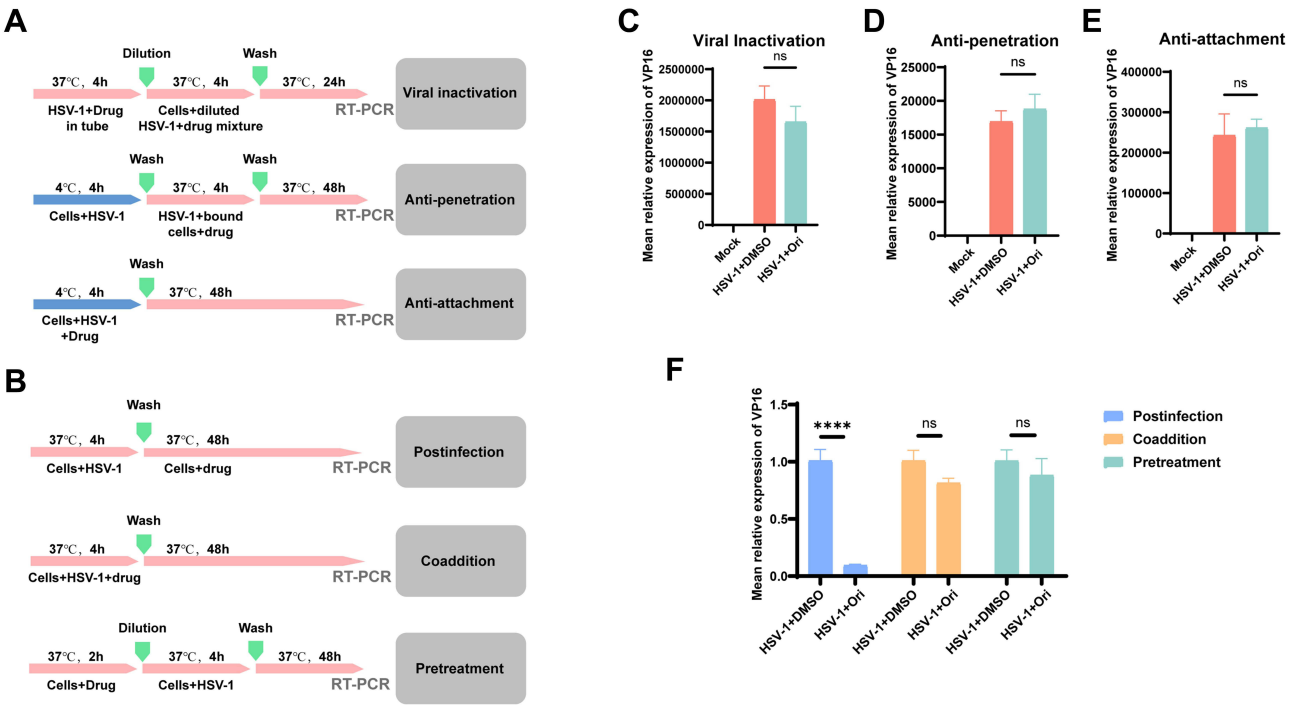

Figure 4 Ori inhibits on HSV-1 virus replication in vitro. (**A**) A schematic of different Ori treatments administered to HSV-1 and Vero cells. Ori treatment in different modes was used to study its effect on the stage of viral inactivation, penetration and attachment. (**B**) A schematic of different Ori treatments administered to HSV-1 and host cells. Ori treatment in different ways was used to study its effect on the pre-infection, coaddition, and the post-infection stage. (**C**–**E**) Ori treatment during viral inactivation, attachment and penetration in the early stage of the HSV-1 life cycle did not disrupt HSV-1 replication. Vero cells were treated with Ori (6 μM) as described in (**A**) and infected with HSV-1. The expression levels of intracellular HSV-1 VP16 were normalized to RPL5. (**F**) Ori treatment post infection repressed HSV-1 virus replication. Vero cells were treated with Ori (6 μM) as described in (**B**) and infected with HSV-1. The expression levels of intracellular HSV-1 VP16 were in each group normalized to RPL5. (****P < 0.0001, ns means no significance). P values were calculated with Student's *t*-test.

principal component analysis (PCA) score plot (Figure 5B), PCA attained good separation between samples in HSV-1 +DMSO, HSV-1+Ori and Mock groups. The PCA revealed that the cumulative contribution rate reached 85.02%. The compact aggregation of the repeated samples showed that the organized model was useful. In the Ori-treated group, 279 genes with fold changes ≥ 2.5 were downregulated (Figure 5C). DEGs enriched in inflammation and immune regulation pathways are presented in the heatmap (Figure 5D). Gene ontology (GO) analysis has been developed to be a useful tool to reveal biologic functions underlying observed patterns in genomics. GO analysis revealed that the downregulated genes were significantly enriched in immune response, angiogenesis, and inflammatory response (Figure 5E). These data suggested that alleviating HSK severity of Ori treatment was strongly associated with the inflammatory response and immune regulation.

Oridonin Can Suppress NLRP3-IL-β-Inflammasome Activation

HSV-1-infected mouse model exhibited reduced expression levels of *nlrp3* at 3 dpi in RNA-seq assay after treatment with Ori. The following qPCR assay indicated that the expression of *caspase 1, il-1β* and *nlrp3* in corneas was significantly reduced in HSV-1+Ori mice than in HSV-1+DMSO controls: 25-fold decrease for *caspase 1*, 3-fold decrease for *il-1β*, and 5-fold decrease for *nlrp3* (Figure 6). Given the effect of IL-18 and cleaved IL-1β on exacerbating inflammation, these results imply that inhibition of NLRP3 by Ori alleviates the inflammation associated with HSK by suppressing the release of cleaved IL-1β.

Discussion

HSV-1 can cause many health problems, including HSK, acute retinal necrosis,[44] encephalitis, and oral lesions. Although many vaccine techniques are being explored, there is currently no HSV-1 vaccine approved by FDA for human use. The frequent and long-term use of anti-herpes drugs, such as ACV and other nucleoside analogs, can exacerbate the

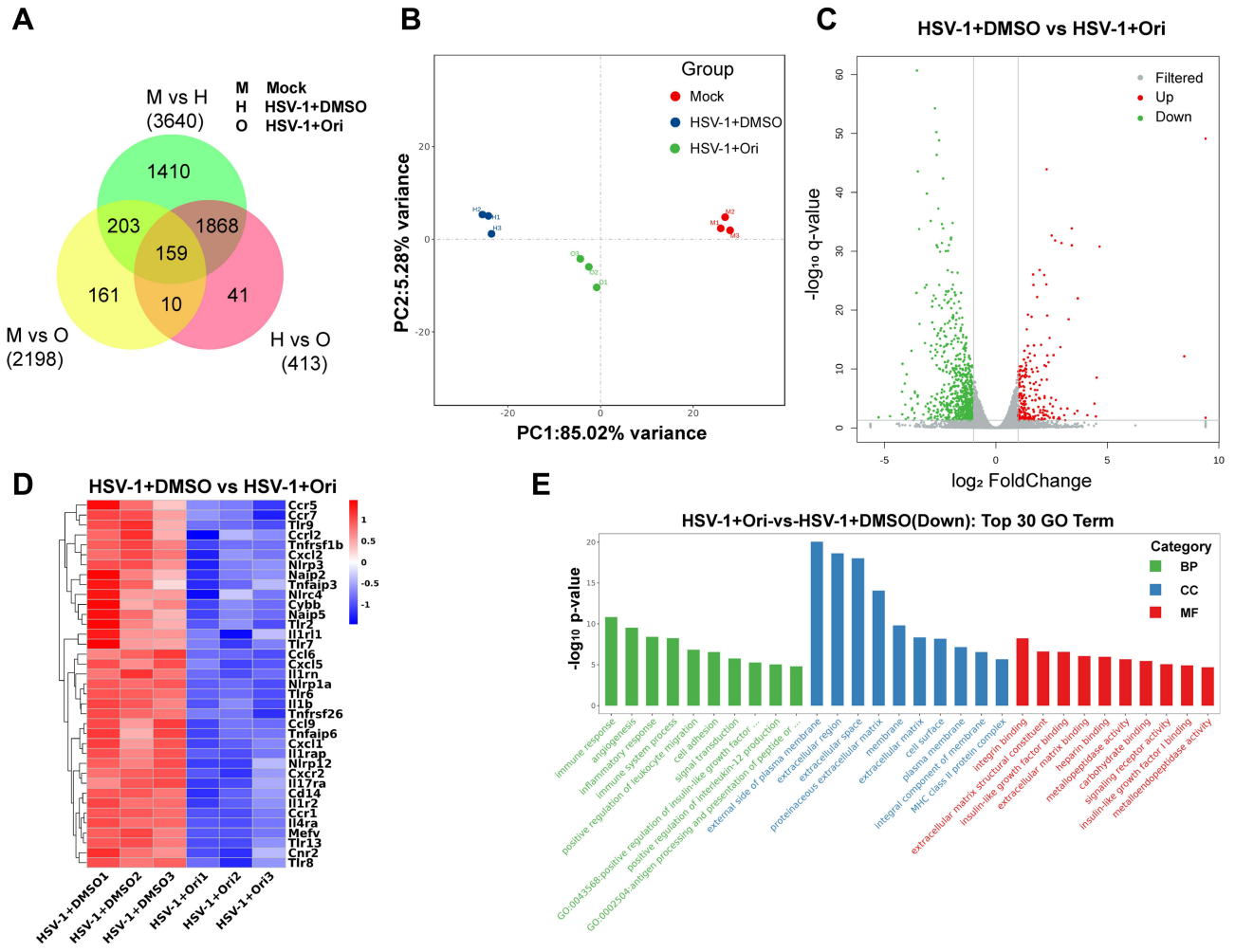

Figure 5 RNA sequencing analysis. (**A**) Venn graph. Co-expressed and uniquely expressed genes in Mock, HSV-1+DMSO and HSV-1+Ori group. (**B**) PCA plot Principal component analysis of Mock, HSV-1+DMSO and HSV-1+Ori samples. (**C**) Volcano Plot of DEGs in HSV-1+DMSO and HSV-1+Ori group. Red dots: upregulated genes with log2 FC > 3.32, and adjusted P < 0.05. The Green dots: downregulated genes with log2 FC < −3.32 and adjusted P < 0.05. FC, fold change; DEGs, differentially expressed genes. (**D**) Heatmap of genes enriched in the GO terms with the higher enrichment factor in HSV-1+DMSO and HSV-1+Ori group. The $\log_{10}$ FC values were used to generate heatmaps. Each row represents a gene in the figure. Red, high expression, and blue, low expression in the group, respectively. (**E**) GO enrichment analysis. GO analysis of target genes for biological processes revealed that downregulated genes were greatly enriched in immune response, angiogenesis, and inflammatory response.

emergence of drug-resistant strains and side effects, especially in immunocompromised patients. Therefore, exploiting new drugs remains an important means of curing HSK. It's confirmed in our study that Ori could ameliorate HSV-1 corneal infection severity in mouse models and revealed that HSV-1 replication could be suppressed by Ori in vivo and in vitro. We also revealed that the relief of HSK effects of Ori may be attributed to its anti-inflammatory effect and inhibitory effect on HSV-1 replication.

Our study implied that the mechanism by which Ori inhibits HSV-1 was different from that of ACV. ACV is the world's first clinically applied compound for treating HSV-1. ACV relies on the activity of the viral enzyme thymidine kinase (TK) to convert ACV to the triphosphate form, which subsequently interferes with viral DNA replication.[45] ACV resistance may appear and engender serious challenges to therapy during primary HSV-1 infection or relapse. A higher probability of ACV resistance has been reported in immunocompetent HSK cases.[46,47] To overcome this problem, some studies highlighted the necessity of researching innovative antiviral drugs.[48] As a new anti-HSV-1 drug, Ori may be a choice to inhibiting ACV-resistant (ACVr) strains. However, we did not conduct further assays due to the lack of ACVr virus strains. With the application of ACV, the side effects of ACV have gradually begun to be paid attention to. Antiviral drugs such as ACV, indinavir, and foscarnet can cause acute kidney injury.[49] Some studies have focused on the neurologic side effects of ACV.[50] We showed that ACV combined with Ori could yield a greater effect on inhibiting

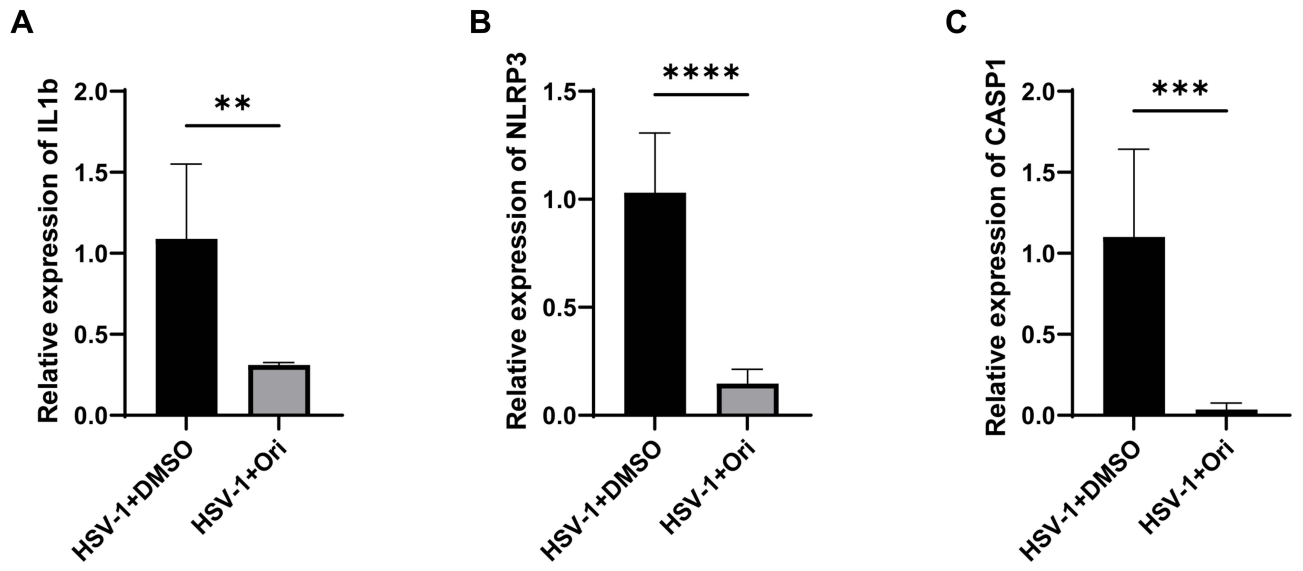

Figure 6 Ori relieving HSK in mice models via suppressing inflammasome. (**A**–**C**) The relative expressions of *il-1β, casp1* and *nlrp3* (n=3) in HSV-1 infected corneas from Mock (0.1%DMSO) or Ori treatment group. (** P < 0.01, *** P < 0.001, **** P < 0.0001, compared with Mock group). *mrpl5* was used as the reference gene. P values were calculated with Student's *t*-test.

HSV-1 infection than ACV alone, which may be due to their different mechanisms. Accordingly, reducing the dose of ACV by combination with Ori may be an appropriate way to reduce the side effects of ACV.

Although Ori injection has been experimentally investigated for years, more preclinical studies should be conducted before clinical therapy of HSV-1 infection treated with Ori. However, Ori exhibits limited efficacy as a therapeutic agent against HSV-1, given its lower solubility and high first-pass elimination in medical applications. The product of Ori nanosuspension and nanoparticles has been utilized to significantly improve its absorption and prolong its onset time,[51] which can also provide new ways of administering, such as subconjunctival injection or intravitreal injection. Other possible methods, such as the synthesis of derivatives of Ori, are available.[19]

Ori can suppress the HSV-1 replication in vitro. Ori treatment was carried out at each single stage of HSV-1 infection to interpret the anti-viral mechanism. The qPCR assay further supported that pretreatment and coaddition of HSV-1 infected Vero cells with Ori exhibited a poor inhibitory effect. However, Ori treatment at the post-infection stage yielded a noticeable suppressing effect on HSV-1 replication. This finding indicated that Ori most likely effected on the post-infection stage of HSV-1 infection.

To determine how Ori alleviating HSV-1 infecting in vivo, we carried out RNA-sequencing, which showed that Ori regulated immune response and inflammatory response in HSK models. We found that Ori reduced the expression of NLRP3 and IL-1beta-inflammasome in the following qPCR assay. Pathogen-associated molecular patterns (PAMPs) existing in pathogens and danger-associated molecular patterns (DAMPs) arising from cell damage are recognized by pathogen recognition receptors (PRRs).[52] This recognition process activates inflammasome which is a supramolecular protein complex by a lysosomal destabilization mechanism.[53]

The assembly of NLRP3 inflammasome which is the most studied among over 20 types of inflammasome results in the activation of caspase-1. Then caspase-1 promotes the cleavage of pro-IL-1β and pro-IL-18 to produce functional IL-1β and IL-18. Another study suggested that HSV-1 infection in cornea could motivate concurrent early expression of the NLRP3 inflammasomes, followed by Caspase-1 activation which provoke the recruitment of neutrophils and inflammatory macrophages into the infected cornea.[54] This excessive inflammatory response induced by HSV-1 infection in cornea leads to HSK, with irrespective of virus replication level. Optimal activation of inflammasomes may arouse a subclinical inflammatory response which does good to the cornea. However, excessive inflammation could harm cornea.[55] Accordingly, dysregulation or hyperactivation of inflammasomes can increase inflammation that aggravates HSK. Our

study focused on the ability of Ori to alleviate the NLRP3-dependent inflammation in the HSK mice model caused by HSV-1 infection.

However, it should be noted that Ori may suppress NLRP3 expression by other pathways. RNA-seq showed that Ori regulated the expression of MAPK or ERK pathway and reduced ROS generation. The overwhelming evidence substantiates that herbs can inhibit the replication of the HSV-1 virus by regulating the MAPK or ERK pathway.[56–58] It is well-established that the generation of ROS is crucial in the pathogenesis of HSK to elevate HSV-1 replication.[59,60] Our study revealed that Ori impaired primary HSK infections in mice, but it was still unclear if Ori will alleviate signs of recurrent HSK. Our future studies will focus on identifying other pathways of Ori and alleviation of recurrent HSK in a mouse model.

Conclusion

This study confirms that Ori can effectively inhibit HSV-1 virus replication in vivo and in vitro; Ori treatment can significantly relieve corneal infection symptoms in the HSK mice model. Ori has potential promise for application in treating HSK and other diseases caused by HSV-1 infection alone or in combination with other therapeutics like ACV.

Data Sharing Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Disclosure

The authors report no conflicts of interest in this work.

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