Inhibition of cyclophilin A suppresses H₂O₂-enhanced replication of HCMV through the p38 MAPK signaling pathway

Jun Xiao[1,2], Xin Song[1,2], Jiang Deng[1,2], Liping Lv[1,2], Ping Ma[1,2], Bo Gao[1,2], Xipeng Zhou[1,2], Yanyu Zhang[1,2] and Jinbo Xu[1,2]

1 Beijing Institute of Transfusion Medicine, China
2 Beijing Key Laboratory of Blood Safety and Supply Technologies, China

Human cytomegalovirus (HCMV) infection can be accelerated by intracellular and extracellular hydrogen peroxide (H₂O₂) stimulation, mediated by the activation of the p38 mitogen-activated protein kinase (MAPK) pathway. However, it remains unknown whether host gene expression is involved in H₂O₂-upregulated HCMV replication. Here, we show that the expression of the host gene, cyclophilin A (CyPA), could be facilitated by treatment with H₂O₂ in a dose-dependent manner. Experiments with CyPA-specific siRNA, or with cyclosporine A, an inhibitor of CyPA, confirmed that H₂O₂-mediated upregulation of HCMV replication is specifically mediated by upregulation of CyPA expression. Furthermore, depletion or inhibition of CyPA reduced H₂O₂-induced p38 activation, consistent with that of H₂O₂-upregulated HCMV lytic replication. These results show that H₂O₂ is capable of activating ROS-CyPA–p38 MAPK interactions to enhance HCMV replication.

Human cytomegalovirus (HCMV) is a widespread pathogen that establishes a lifelong persistent infection and causes life-threatening symptoms in immunocompromised or immunosuppressed hosts [1]. There are three drugs currently approved for HCMV treatment: ganciclovir and its prodrug valganciclovir, foscarnet, and cidofovir [2]. However, the utility of each has been limited by significant toxicity. Since combinatorial treatments are not currently being considered due to their cytotoxicity, therapies with diverse mechanisms of action are highly desirable. Earlier work from our laboratory [3] and others [4,5] has demonstrated that oxidative stress could upregulate HCMV replication, and identify antioxidants as a possible target for treatment of HCMV infection. However, the underlying mechanism linking oxidative stress and HCMV replication remains poorly characterized.

Cyclophilin A (CyPA) was originally discovered as a cellular factor with high affinity for the immunosuppressant cyclosporine A (CsA) [6]. Previous studies have demonstrated that ischemia/reperfusion (I/R) and hypoxia can induce expression of CyPA [7–9]. CyPA can be also secreted from monocytes/macrophages [10], endothelial cells [11], and vascular smooth muscle cells [12] in response to ROS. Elevated CyPA have been reported in association with various viral infections. Studies have shown that HIV-1 replication was reduced in human CD4+ T cells when CyPA was knocked out [13]. Thus, these studies suggest CyPA plays a significant role in promoting HIV infection.

Abbreviations

CsA, cyclosporine A; CyPA, cyclophilin A; H₂DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HCMV, human cytomegalovirus; HDACs, histone deacetylases; HFF, human foreskin fibroblast; IE1, immediate early; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; NAC, N-acetylcysteine; PPIase, peptidylprolyl-cis–trans-isomerase; ROS, reactive oxygen species; TCID₅₀, 50% tissue culture infective dose; VSMC, vascular smooth muscle cells.

CyPA was reported to bind HIV-1 Gag polyprotein in a Cs-sensitive complex [14], which is essential for HIV replication [15]. Recent studies have reported that CypA induces a repressive effect on the replication of some viruses, including influenza A virus [16], rotavirus [17], and HEV [18].

CyPA has also now been understood to represent a key factor in the regulation of cytomegaloviruses, modulating replication of murine cytomegalovirus (MCMV) and HCMV [19,20]. During MCMV infection in NSPC (Neural stem/progenitor cells), CyPA has been suggested to play an important role in regulation of major immediately early promoter (MIEP) chromatin modification by interacting with histone deacetylases (HDACs). Despite the enormous potential for CyPA in modulating viral response, the role of CyPA during ROS-upregulated HCMV replication remains largely uncharacterized. In this study, we examined whether CyPA participates in hydrogen peroxide (H_2O_2)-mediated cytomegalovirus (CMV) replication and its mode of action.

Materials and methods

Cell culture, chemical reagents and antibodies

Human foreskin fibroblast (HFF) cells, mouse embryonic fibroblast (MEF) cells of no more than 15 passages, and HEK 293 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37 °C under a 5% CO_2 atmosphere [3].

H_2O_2 solution, *N*-acetylcysteine (NAC), CsA, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), *N*-succinyl-Ala-Ala-Pro-*p*-nitroanilide, α-Chymotrypsin, and the p38 inhibitor SB203580 were purchased from Sigma Life Science (St. Louis, MO, USA).

Rabbit polyclonal or monoclonal antibodies used in this study included phospho-p38 (T180/Y182), p38, CyPA, and β-actin were provided by ABclonal technology (Cambridge, MA, USA) and the mouse monoclonal antibodies to HCMV pp72 and pp65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) [3].

Viral preparation and titration

Human cytomegalovirus (AD169strain) and MCMV (Smith strain) stocks were prepared in HFF cells and MEF cells, and aliquots were stored at −80 °C. HCMV was used to infect cells at a multiplicity of infection (MOI) of 0.5 for all of the current experiments. Viral titers were detected using the 50% tissue culture infective dose ($TCID_{50}$) assay, as previously described [3,21]. All experiments were examined at least three times using Reed and Muench's method.

Generating stable CyPA knockdown cell lines

Lentiviral vectors, targeting cyclophilin A (siCypA) or a random sequence (siCTR), were produced by Hanbio Co. Ltd (Shanghai, China). The siRNA sequences were: CyPA, 5′-GATCCGTGGTGACTTCACACGCCATAATTCAAGAGATTATGGCGTGTGAAGTCACCATTTTTTC-3′. HFF and HEK293 cells were infected at an MOI of 100 or 10, respectively. The siRNA recombinant lentivirus was incubated with 8 $\mu g \cdot mL^{-1}$ polybrene to enhance the lentivirus infection. For the stable knockdown cell lines, the HFF or HEK293 cells were incubated in a selection medium containing 2 $\mu g \cdot mL^{-1}$ puromycin (Invitrogen, Carlsbad, CA, USA) beginning 48 h after transduction.

Dichlorofluorescein staining

Dichlorofluorescein staining was operated as previously described with slight modification [3]. Cells were seeded on 24-well culture plate, stimulated with H_2O_2 (200 μM) for 24 h or infected with MCMV for 72 h and then incubated with H_2DCF-DA (10 μM) in serum-free DMEM for 0.5 h at 37 °C (dark conditions). Cells were then washed with PBS three times and images were taken by Leica microscope (Wetzlar, Germany).

Luciferase assays

In 24-well plates, 10^5 cells per well were cultured to confluence and were transiently transfected with the MIEP-pGL3 reporter plasmid and the pRL-TK vector. Twelve hours following transfection, the cells were treated with the CsA (1 μM) for 1 h and then stimulated with H_2O_2 (200 μM) for 12 h. Luciferase activity was measured as previously described [3,22] using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Western blot analysis

Western blot analysis was carried out as previously described [23]. Proteins in cell lysates were heated for 5 min at 95 °C, and loaded onto a 12% SDS/PAGE gel. Proteins were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and incubated with indicated antibodies. Proteins bands were visualized using western blotting luminol reagent according to the manufacturer's protocol (Santa Cruz Biotechnology). The membranes were incubated with western blot stripping buffer (CWBio, Beijing, China) to reprobe for other proteins on the same membrane.

CMV genome quantification

Total DNA was isolated as previously described [3]. Viral DNA was quantified using quantitative PCR (qPCR) on a CFX-96 thermocycler (Bio-Rad, Hercules, CA, USA), using

CMV specific primers. Primers used are 5′-ATGTACGGG GGCATCTCTCT-3′ (forward) and 5′-GGCTTGGTTATC AGAGGCCG-3′ (reverse) for HCMV genome or the MC MV genomic primer, 5′-GTGGGCATGAAGTGTGGGTA-3′ (forward) and 5′-CGCATCGAAAGACAACGCAA-3′ (reverse).

Real-time quantitative PCR

The real-time quantitative PCR (RT-qPCR) method was described previously [3]. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen) 24 h following HCMV infection (MOI = 0.5). Approximately 500 ng of RNA was transcribed into cDNA using ReverTra Ace® qRCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Each sample was measured in triplicate. The HCMV IE1 expression level (forward primer, 5′-GTTGGCCGAA GAATCCCTCA-3′ and reverse primer, 5′-CACCATGTC CACTCGAACCT-3′) and human CyPA gene transcript (forward primer, 5′-GCTGGACCCAACACAAATGG-3′ and reverse primer, 5′-GCTCCATGGCCTCCACAATA-3′) were normalized to GAPDH mRNA (forward primer, 5′-CATGAGAAGTATGACAACAGCCT-3′ and reverse primer, 5′-AGTCCTTCCACGATACCAAAGT-3′). The expression of mouse CyPA gene transcript (forward primer, 5′-AAAGCATACAGGTCCTGGCATC-3′ and reverse primer, 5′-CATGCTTGCCATCCAGCCAT-3′) was normalized to mouse GAPDH mRNA (forward primer, 5′-CCGT CGTGGATCTGACGTG-3′ and reverse primer, 5′-GGTC CTCAGTGTAGCCCAAG-3′). Compared to the untreated cells or uninfected mice, the relative expression levels in treated cells and infected mice were calculated as fold changes.

Animal studies

BALB/c mice (male, 3–4 weeks old, and 15–20 g body weight) were purchased from Vital River (Beijing, China). The protocols used in this study were approved by the Ethics Committee at the Beijing Institute of Transfusion Medicine and were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Mice were treated with vehicle (olive oil) or 10 mg·kg^{-1}·day^{-1} CsA by gavage, from 3 days before intraperitoneal inoculation with MCMV (Smith strain, 5×10^3 p.f.u). At day 7, 14, 21 and 28 post infection, total DNA was extracted from 100 μL whole blood and used to detect the viral DNA genome. To measure infectious virions in mice organs, the salivary glands (50 mg) and the lung (50 mg) were collected on day 14 and 28 post infection and homogenized, then viral titer was calculated with TCID$_{50}$ assays in MEF monolayers.

CyPA activity assay

The CyPA activity was detected as previously described [24]. The *cis–trans* isomerization of Ala-Pro peptide bond in the test peptide *N*-succinyl-Ala-Ala-Pro-*p*-nitroanilide (100 μM) was measured in an assay with α-Chymotrypsin (10 μM). Briefly, reactions were at 15 °C and contained 30 μL of test peptide, 0.1 M Tris-HCl (pH 7.8) and 100 μg test sample. After incubation for 1 min, 30 μL of 2 mg·mL^{-1} α-Chymotrypsin in 0.1 M Tris-HCl was added. After mixing, the absorbance was detected at 390 nm.

Statistical analyses

Statistical analyses were carried out using previously described methods [3]. All values are expressed as the means ± standard deviations. Statistical analyses were performed using SPSS statistical software V.17 (SPSS Inc., Chicago, IL, USA). Significant differences were evaluated by two-tailed Student's *t*-test when two groups were compared, one-way analysis of variance (ANOVA) followed by the Dunnett's test when multiple groups were tested against a control group and the Bonferroni *post hoc* test when performing multiple comparisons between groups. A *P*-value less than 0.05 was considered as a statistically significant difference.

Results

H$_2$O$_2$ induces upregulation of cyclophilin A

To investigate whether CyPA can be enhanced in HFF cells by H$_2$O$_2$ stimulation, RT-qPCR analysis was performed using mRNA extracted from HFF cells exposed to H$_2$O$_2$. Firstly, the effect of H$_2$O$_2$ on cell death was detected by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay (Fig. S1). Then, an increase in CyPA transcripts levels were observed at 50 μM H$_2$O$_2$ and continued to increase up to a concentration of 200 μM (Fig. 1A). These findings were also recapitulated at the protein level (Fig. 1B). To confirm these results, we repeated the analysis using an antioxidant, *N*-acetylcysteine (NAC), to counteract the effects of H$_2$O$_2$. Although increasing CyPA mRNA and protein levels were observed following exposure to 200 μM H$_2$O$_2$ (Fig. 1C,D), a significant decrease in CyPA expression was detected in a dose-dependent manner following treatment with NAC (Fig. 1C,D). These results indicated that H$_2$O$_2$ could enhance the expression of CyPA in a dose-dependent manner in HFF cells.

Silencing CyPA in HFF cells prevents H$_2$O$_2$-upregulated HCMV replication

CyPA has been known to play an important role in viral replication [25]. To assess the effect of the CyPA on H$_2$O$_2$-enhanced HCMV replication, we performed a luciferase assay to investigate the activation of HCMV MIE promoter after cells were stably
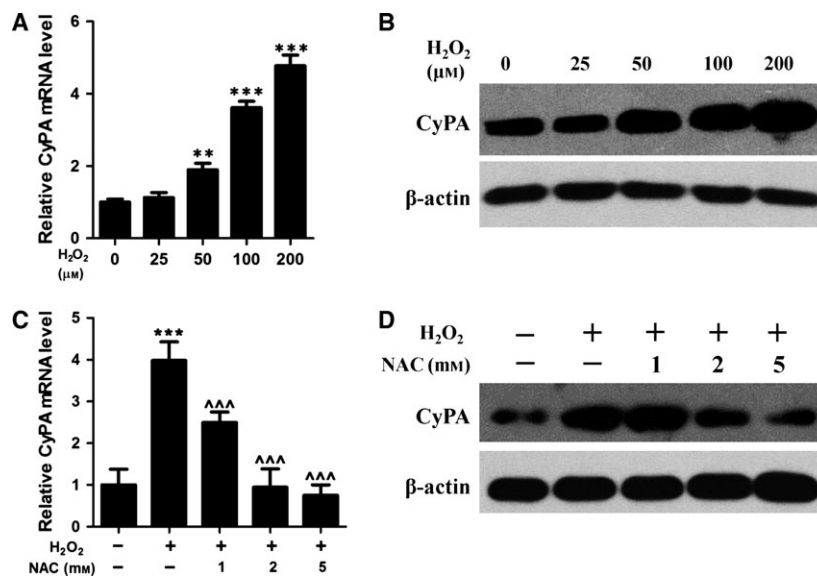
Fig. 1. Cyclophilin A (CyPA) was induced in human fibroblast cells treated with H_2O_2. After supplementation with 0, 25, 50, 100, 200 μM H_2O_2 for 24 h, increasing CyPA levels in human foreskin fibroblast (HFF) cells were observed by RT-qPCR (A) or by western blotting (B). Treatment of HFF cells with 0, 1, 2, 5 mM H_2O_2 scavenger *N*-acetylcysteine (NAC) prior to H_2O_2 (200 μM) inhibited the H_2O_2-induced CyPA expression (C) and protein changes (D). The data are expressed as the means ± SD. **$P < 0.01$ or ***$P < 0.001$ compared with control HFF cells and ^^^$P < 0.001$ versus H_2O_2-treated cells.

transfected with siCTR or siCyPA and exposed to H_2O_2 for up for 12 h. Before H_2O_2 treatment, the baseline expression levels of CyPA were evaluated in transfected cells by western blotting. Compared with wild-type cells, the expression of CyPA was reduced by approximately 90% following siCyPA interference (Fig. S2). CyPA knockdown resulted in a reduction of luciferase activity as compared to control cells in the context of H_2O_2, suggesting that CyPA is involved in H_2O_2-upregulated MIEP activity (Fig. 2A). Next, we tested whether HCMV gene transcripts and protein expression were affected by CyPA interference in HFF. Consistent with the luciferase assay results, silencing of CyPA resulted in downregulation of H_2O_2-enhanced CyPA and HCMV IE1 expression (Fig. 2B) and HCMV replication (Fig. 2C,D). Furthermore, the production of infectious virions was elevated with H_2O_2 in siCTR HFF cells while depletion of CyPA reversed this effect (Fig. 2E). These results indicate that CyPA plays an important in H_2O_2-upregulated HCMV replication.

## The activity of CyPA is required for H_2O_2-upregulated HCMV replication

CyPA is the major intracellular receptor of CsA which is known to bind to and inhibit CyPA activity [26]. However, the effect of CsA on the H_2O_2-upregulated

CyPA expression has not yet been fully understood. The results presented here demonstrated that CsA exhibited little effect on H_2O_2-upregulated CyPA gene and protein expression (Fig. S3A), as well as H_2O_2-induced ROS (Fig. S3B). To further characterize the influence of CsA on HCMV replication, we evaluated MIEP activity by luciferase assay. The results revealed that H_2O_2 stimulated the activity of MIEP, an effect that could be reversed by treatment with CsA (Fig. 3A). Taken together, these results suggested that CsA could hinder the activity, but not the expression of CyPA, thereby resulting in the inhibition of H_2O_2-enhanced viral replication.

To evaluate the antiviral effect of CsA, we evaluated the expression level of viral and host genes. In the presence of H_2O_2, viral IE1 mRNA levels were downregulated by treated with CsA, whereas CyPA mRNA levels were not impacted (Fig. 3B). To confirm these findings, we performed western blotting to detect CyPA and viral pp72 and pp65 proteins in infected cells treated for 5 days with CsA. pp72 and pp65 were downregulated by supplementing with CsA, while CyPA expression was unaffected (Fig. 3C).

To examine the effect of CyPA activity on the full cycle of viral replication, real-time PCR and viral titration were performed to detect viral DNA changes and infectious virion production. Treatment with H_2O_2 produced higher viral DNA load and viral titer as
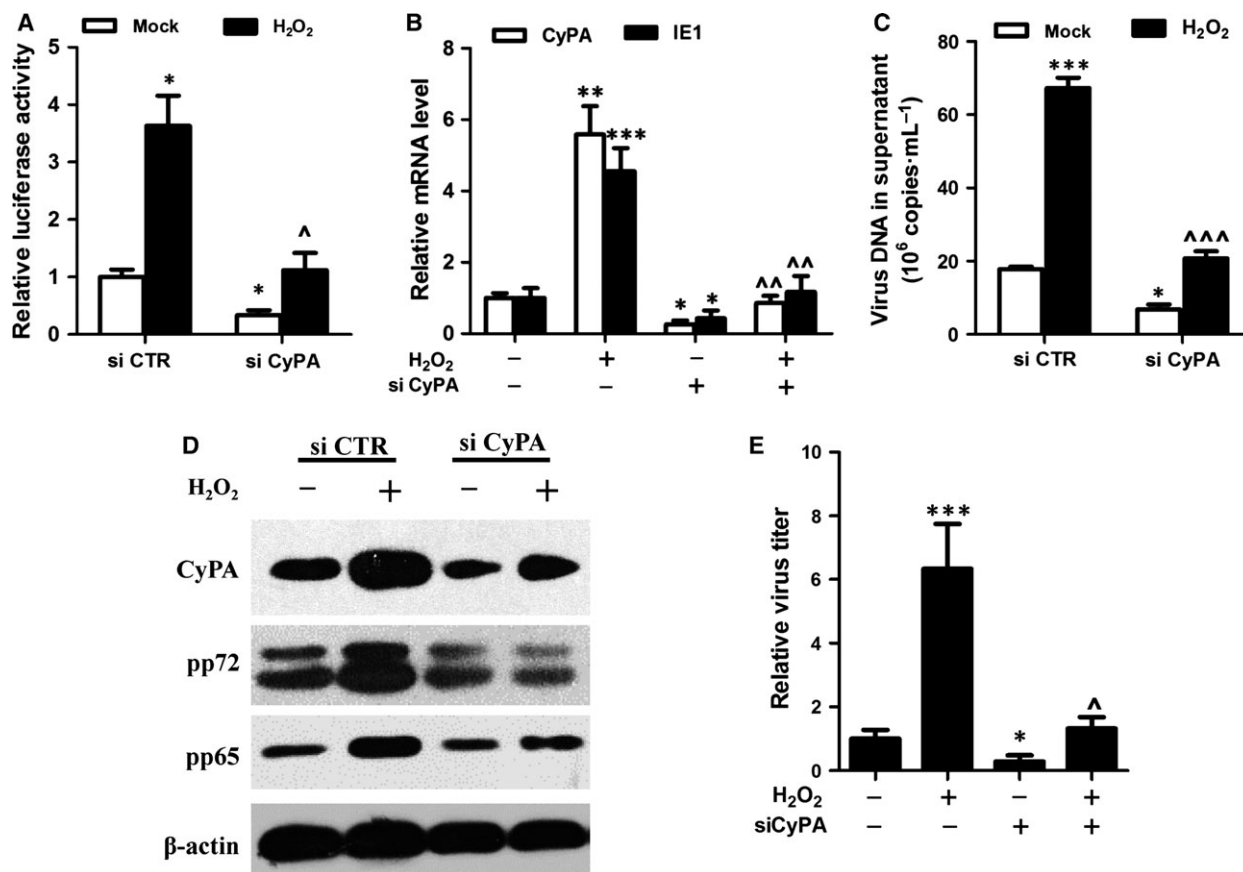
Fig. 2. Depletion of CyPA protein delayed H_2O_2-upregulated viral replication. Luciferase activities were measured in siRNA-293 cells, stably expressing siCTR or siCyPA. These cells were transfected with MIEP-pGL3 plasmid for 24 h and treated with 200 μM H_2O_2 for 12 h (A). Human foreskin fibroblast (HFF) siCTR and siCyPA cells were infected at a Multiplicity of Infection (MOI) of 0.5 for 24 or 72 h. Treated with 200 μM H_2O_2 for 24 h upregulated CyPA and human cytomegalovirus (HCMV) IE1 transcription in HFF siCTR cells, but the effect was attenuated in HFF siCyPA cells (B). The virions in the cultural supernatant and pp72 and pp65 viral proteins in HCMV infected (MOI = 0.5) HFF siCTR and siCyPA cells treated with H_2O_2 (200 μM) for 72 h were determined by qPCR and western blotting (C, D). HFF siCTR and siCyPA cells were infected with HCMV (MOI = 0.5) in the presence of H_2O_2 (200 μM) for 5 days, and monolayers were analyzed by viral titration (E). $*P < 0.05$; $**P < 0.01$ or $***P < 0.001$ for treated versus mock treated cells or HFF siCTR cells. $^P < 0.05$; $^^P < 0.01$ or $^{^{^}}P < 0.001$ for HFF siCyPA versus HFF siCTR under the treatment with H_2O_2.

compared to the control group, while CsA decreased H_2O_2-upregulated HCMV lytic replication (Fig. 3D, E).

Effect of CsA on the inhibition of MCMV replication *in vivo*

The results mentioned above revealed that CyPA acted as a modulator in H_2O_2-enhanced viral replication *in vitro*. In order to further confirm this effect, we investigated the antiviral effect of CsA on viral replication *in vivo*. The result showed that primary infection of MCMV could induce ROS generation in MEF cells (Fig. 4A). This indicated that the induced ROS might stimulate the expression of CyPA to expand the viral

production. After being infected with MCMV for 14 days, higher expression of CyPA was exhibited in the salivary gland and the lung in infected mice (Fig. 4B). Thus, we considered whether CsA could inhibit the viral replication during the primary infection *in vivo*.

Mice were treated with vehicle (olive oil) or 10 mg·kg^{-1}·day^{-1} CsA by gavage, 3 days before intraperitoneal inoculation with MCMV (Smith strain, 5×10^3 p.f.u). On days 7, 14, 21 and 28 post infection, viral DNA was extracted from whole blood to explore the impact of CsA treatment. The results demonstrated that CsA treatment significantly decreased the viral DNA load during primary infection (Fig. 4C). Additionally, the viral titration results confirmed the effect of CsA. When compared against
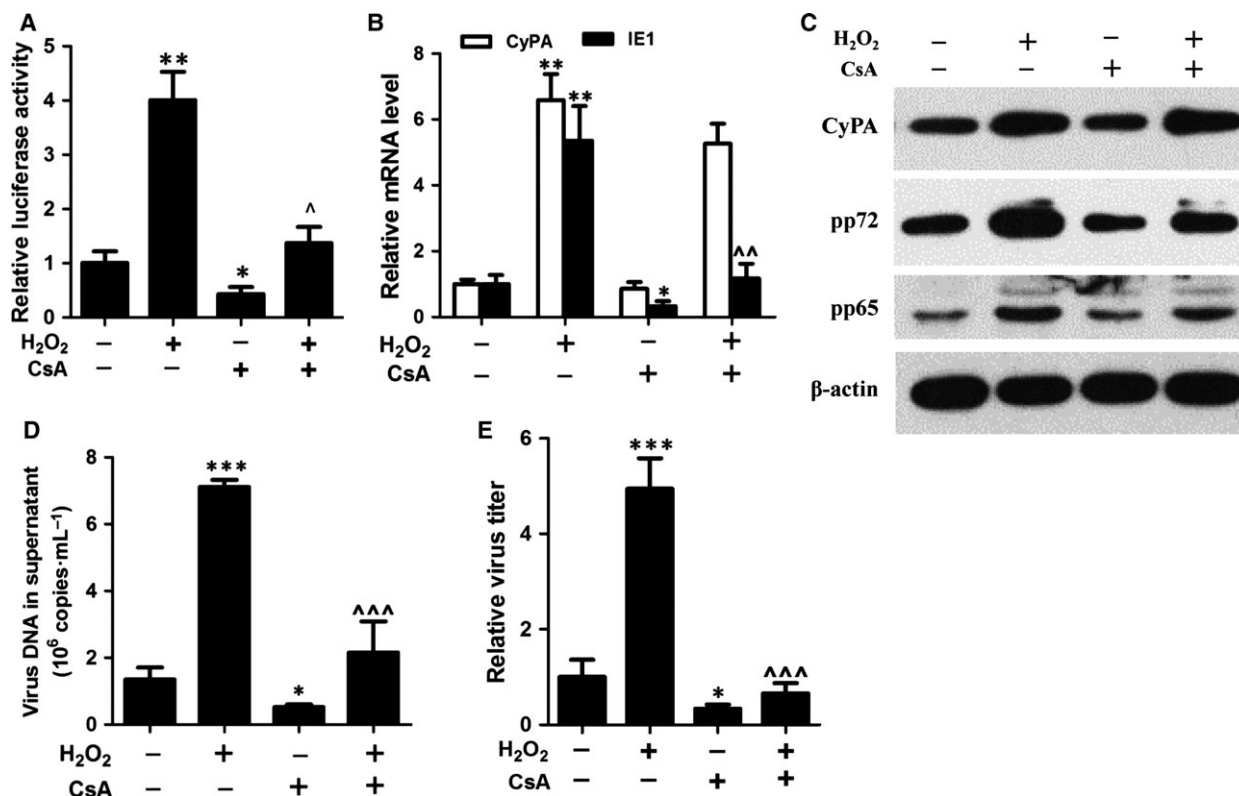
Fig. 3. Inhibition of CyPA activity prevents H_2O_2-enhanced human cytomegalovirus (HCMV) replication in human foreskin fibroblast cells. Luciferase activities were detected in HEK 293 cells which were transfected with MIEP-pGL3 plasmid for 24 h and treated with 1 μM cyclosporine A (CsA) prior to H_2O_2 (200 μM) for 12 h (A). Changes of CyPA and IE1 gene expression was evaluated following treatment with CsA (1 μM) in the presence of 200 μM H_2O_2 (B). The viral protein expression and viral load in the supernatant were detected by western blotting (C) and quantitative PCR (qPCR) (D) in the presence of CsA (1 μM) prior to H_2O_2 (200 μM) treatment for 72 h. Cells treated with CsA (1 μM) in the presence of H_2O_2 (200 μM) were infected with HCMV (MOI = 0.5) for 5 days and were analyzed by $TCID_{50}$ assay (E). *$P < 0.05$; **$P < 0.01$ or ***$P < 0.001$ compared with untreated cells. ^$P < 0.05$ or ^^^$P < 0.001$ versus H_2O_2-treated cells.

controls, CsA treatment resulted in decreased viral production on days 14 and 28 (Fig. 4D). Taken together, these results suggest that CsA treatment might help to prevent CMV replication *in vivo*.

CyPA is involved in H_2O_2-mediated activation of p38-MAPK

It is known that p38-mitogen-activated protein kinase (MAPK) is rapidly and strongly activated by H_2O_2 treatment, in a time- and dose-dependent manner. As CsA treatment inhibited H_2O_2-upregulated viral replication, we hypothesized that the CsA-treatment effect may be related to downstream p38 MPAK pathway activation. As shown in Fig. 5A, inhibition and depletion of CyPA decreased the viral transcription in the context of H_2O_2 stimulation, but unlike siCyPA treatment, CsA and SB203580 has no effect on the expression of CyPA. To confirm the effect of CsA on CyPA, the activity of CyPA was detected. The CyPA activity was increased by

treatment with H_2O_2, while supplementing with CsA decreased the H_2O_2-induced CyPA activity (Fig. 5B). This indicated that inhibition of the H_2O_2-upregulated viral replication may act in a CyPA-dependent manner. Western blotting revealed that depletion and inhibition of CyPA strongly hindered the H_2O_2-stimulated p38 activation (Fig. 5C) and viral proteins expression (Fig. 5D). In keeping with the RT-PCR result, the CyPA expression was also not affected by CsA or SB203580, and the viral titer was strongly inhibited by depletion or inhibition of CyPA (Fig. 5E). These results indicate that CyPA is a critical factor in ROS/p38-MAPK pathway-regulated HCMV replication.

Discussion

Although HCMV replication can be enhanced by treatment with H_2O_2 and can be inhibited by antioxidants, the host-cell molecular interactions involved in H_2O_2-enhanced replication are poorly understood. In
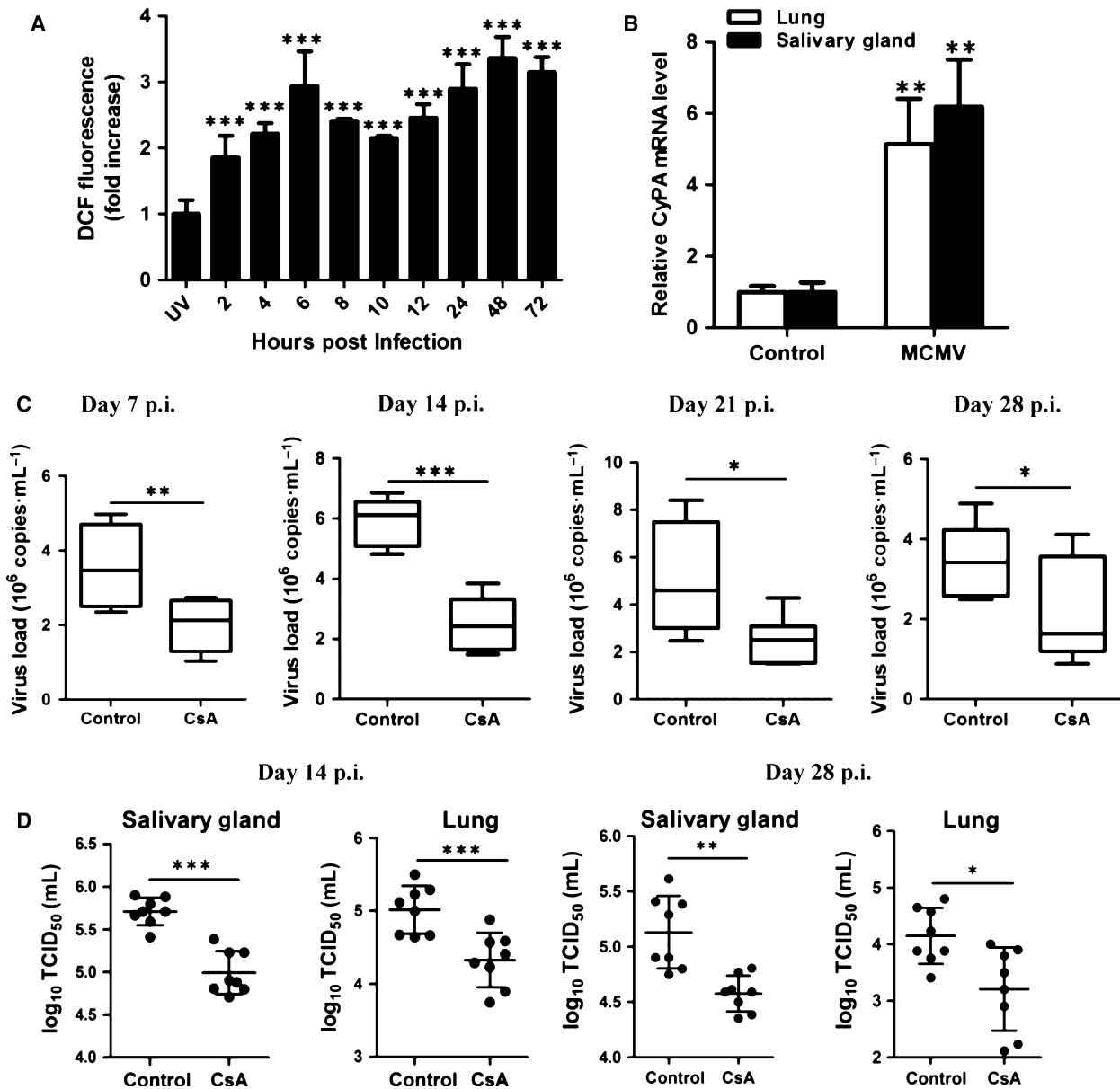
Fig. 4. Cyclosporine A (CsA) treatment inhibited MCMV lytic replication *in vivo*. MEF cells in 24-well plates were infected with either UV-inactivated MCMV or MCMV (MOI = 0.5) and incubated with 10 μM H2DCF-DA for 30 min at 37 °C. The H$_2$DCF-DA fluorescence were detected between infected and uninfected cells at indicated times (A). Fold induction of CyPA expression in infected mice were detected post 14 days MCMV infection (B). Mice were intragastrically treated daily with 10 mg·kg^{-1} CsA, 3 days prior to intraperitoneal inoculation with MCMV (Smith strain, 5 × 10^3 p.f.u). About 100 μL whole blood from each mouse were examined. Viral DNA loads in blood samples of control ($n = 8$) and CsA-treated ($n = 8$) mice at indicated days post infection (C). Related infectious viral titer in the salivary gland and in the lung was detected at day 14 and 28 post infection by TCID$_{50}$ assay (D). *$P < 0.05$; **$P < 0.01$ or ***$P < 0.001$ versus untreated mice.

this study, we demonstrated that H$_2$O$_2$ stimulation could enhance CyPA expression in HFF cells, thus resulting in increased HCMV replication mediated by p38-MAPK pathway activation (Fig. 6).

Cyclophilins are a family of highly conserved and ubiquitous proteins termed immunophilins [27]. The most abundant cyclophilin is CyPA [28], which was

identified as the main target for the immunosuppressive drug CsA [29,30]. CyPA catalyzes the *cis–trans* isomerization of peptidyl-prolyl bonds of cytoplasmic proteins, and acts to promote proteins folding and assembly. Previous studies have indicated that CyPA secretion was stimulated by reactive oxygen species (ROS) in vascular smooth muscle cells (VSMC) [12];
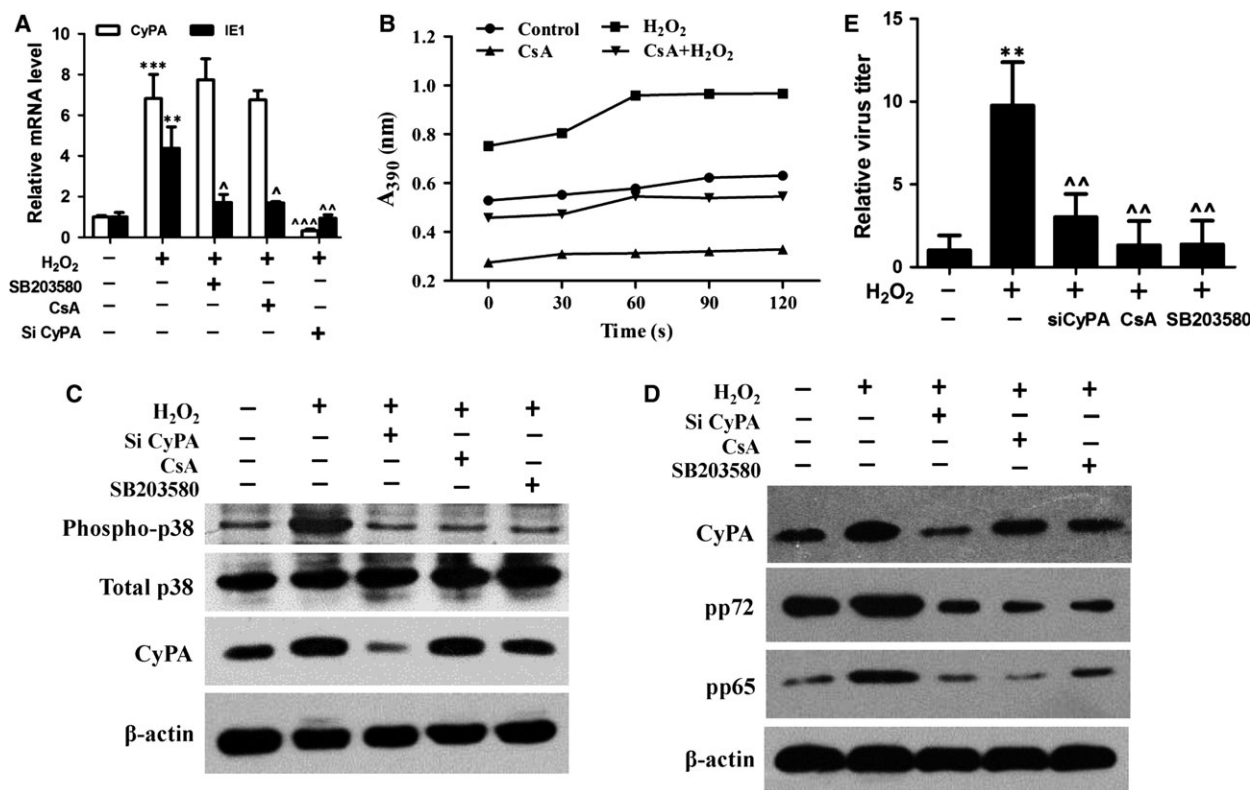
Fig. 5. CyPA was involved in the activation of p38-MAPK pathway during H_2O_2-enhanced human cytomegalovirus (HCMV) replication. Human foreskin fibroblast (HFF) cells and HFF siCyPA cells were treated with SB203580 (10 μM) or cyclosporine A (CsA; 1 μM) 1 h prior to H_2O_2 (200 μM). Cells were infected with HCMV at an MOI of 0.5. Real-time PCR analysis of CyPA and IE1 mRNA levels was performed to compare expression of untreated or H_2O_2-treated cells (A). Cells were treated with CsA (1 μM) prior to H_2O_2 (200 μM) for 24 h and then cells were harvested for CyPA activity assay (B). Activation of p38 was detected in HFF cells and HFF siCyPA cells treated with SB203580 (10 μM) or CsA (1 μM) 1 h prior to H_2O_2 stimulation. Cells were harvested for western blotting 6 h following H_2O_2 treatment (C). Viral proteins were detected in the presence or absence of H_2O_2 (200 μM) under treatment with SB203580 (10 μM) or CsA (1 μM) in HFF cells or HFF siCyPA cells (D) post 72 h HCMV infection. Cells treated with CsA (1 μM) or SB203580 (10 μM) in the presence of H_2O_2 (200 μM) were infected with HCMV (MOI = 0.5) for 5 days and were analyzed by viral titration (E) **$P < 0.01$ or ***$P < 0.001$ versus untreated cells. ^$P < 0.05$; ^^$P < 0.01$ or ^^^$P < 0.001$ compared with H_2O_2-treated cells.

however, this model has been largely uncharacterized in fibroblasts. In the present study, we demonstrated that CyPA expression is induced by H_2O_2 in HFF cells and this effect could be inhibited by the addition of antioxidants.

Several viruses, such as influenza virus, HIV, and HCV, have been reported to induce viral replicatoin in the context of cellular oxidative stress [31–33]. Similarly, the host cellular protein CyPA is also known to be involved in the replication of these viruses [16,34,35]. It has been reported that HCMV infection induces the generation of ROS minutes after entry into the host cell [4]. Furthermore, previous study indicated that oxidative stress could enhance HCMV replication [3,5]. Thus, CyPA may represent a critical factor in mediating the effects of H_2O_2-enhanced HCMV replication. This hypothesis was supported by our results

which demonstrated that knockdown of CyPA resulted in a delay in the H_2O_2-upregulated production of HCMV. CyPA appears to play a crucial role in H_2O_2-upregulated HCMV replication in HFF cells.

Cyclosporine A represents a pharmacological means of inhibiting CyPA activity. Studies have shown that CsA can induce high levels of ROS [36]. However, CsA supplementation prior to H_2O_2 treatment suggested that CsA has no effect on the inhibition of H_2O_2-mediated oxidative stress status and CyPA expression in the present study. This indicates that CsA affects the activity, but not the redox homeostasis and expression of CyPA. CsA supplementation inhibits the MIEP, as well as the viral IE1 gene and protein expression and the production of viral particles in the presence of H_2O_2 without affecting the ROS levels or CyPA expression.
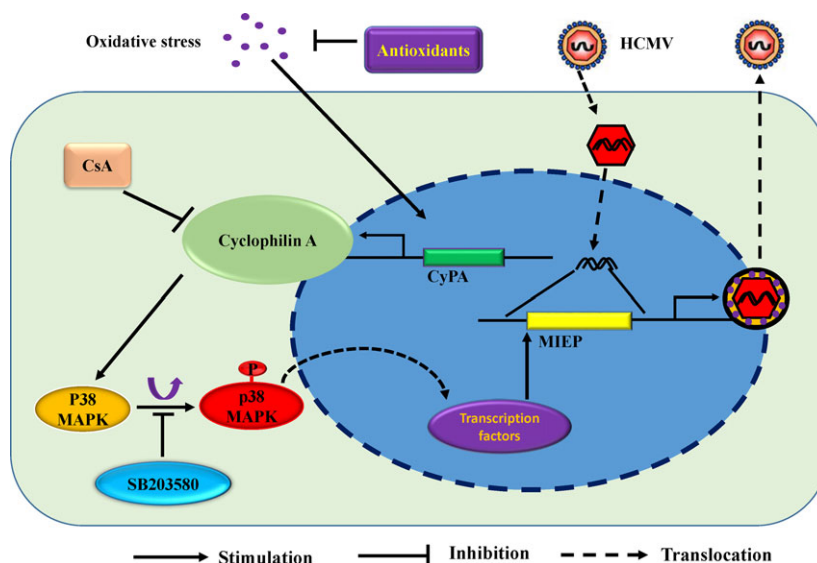
Fig. 6. Model for inhibition mechanism of antioxidants, cyclosporine A (CsA) and SB203580. Oxidative stress could enhance human cytomegalovirus (HCMV) replication though ROS/CyPA/p38-MAPK pathway. However, treatment with antioxidants, CsA or SB203580 to inhibit the molecule involved in this pathway could attenuate the ROS-upregulated HCMV replication.

Although it has been reported that HCMV could induce multiple means to modulate the redox homeostasis [37], HCMV infection can induce oxidative stress *in vitro* as well as an inflammatory response in primary HCMV infection patients [38], suggesting that CyPA may be induced during HCMV infection. This is may be the reason why silencing CyPA could inhibit the HCMV replication in the absence of H_2O_2 [20]. Furthermore, this study has demonstrated that CsA could inhibit MCMV replication in neural stem/progenitor cells while it has little impact in MEF cells [19]. As an immunosuppressive drug, however, it has been reported that CsA could inhibit MCMV infection *in vivo* [39], but the specific mechanism about this phenomenon is not yet clear. In the present study, the oxidative stress status was induced following infection with MCMV and the CyPA gene expression in mice was also enhanced after infection with MCMV. Consistent with previous results, treatment with CsA inhibited the viral DNA load and titer *in vivo*. Taken together our results suggest that CyPA may play an important role in regulating H_2O_2-upregulated viral replication and indicate that the therapeutic method based on CsA or CsA-derived chemicals should be an attractive strategy.

Our previous study [3] demonstrated that the p38-MAPK pathway participates in H_2O_2-upregulation of viral replication. Treatment with CyPA could induce the activation of p38 in KG-1-derived DCs [40], while

other study showed that silencing CyPA could also enhance the activation of p38 [41]. Thus, we have no idea about the relationship between CyPA and the activation of p38. In this study, the p38 inhibitor, SB203880, decreased the viral gene transcription, but rarely affected the H_2O_2-induced CyPA expression in HFF. Depleting and inhibiting CyPA, however, reduced p38 phosphorylation, while SB203580 could not affect H_2O_2-induced CyPA protein expression. This indicates that CyPA regulates the activation of p38, whereas p38 has little effect on H_2O_2-induced CyPA expression. These results suggest a relationship between CyPA and the ROS/p38 MAPK pathway during HCMV infection (Fig. 6). However, the mechanism of how CyPA regulates p38 activation needs further study.

Consequently, we provided evidence that CyPA was associated with the regulation of H_2O_2-induced p38 reactivation during HCMV infection. Thus, targeting of ROS/CyPA/p38-MAPK may be a potential therapeutic or preventive approach in HCMV infection.

Acknowledgements

Author contributions

JX designed, performed experiments, and wrote the paper; JD, and SX performed experiments; PM, LPL, XPZ, and BG performed viral titer experiment; JX and JD analyzed the data; YYZ and JBX gave scientific advices and contributed to a deep manuscript revision. All authors contributed substantially to the present work, then read and approved the final manuscript.

References

1 Crough T and Khanna R (2009) Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev* **22**, 76–98.

2 Biron KK (2006) Antiviral drugs for cytomegalovirus diseases. *Antiviral Res* **71**, 154–163.

3 Xiao J, Deng J, Lv L, Kang Q, Ma P, Yan F, Song X, Gao B, Zhang Y and Xu J (2015) Hydrogen peroxide induce human cytomegalovirus replication through the activation of p38-MAPK signaling pathway. *Viruses* **7**, 2816–2833.

4 Speir E, Shibutani T, Yu ZX, Ferrans V and Epstein SE (1996) Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. *Circ Res* **79**, 1143–1152.

5 Scholz M, Cinatl J, Gross V, Vogel JU, Blaheta RA, Freisleben HJ, Markus BH and Doerr HW (1996) Impact of oxidative stress on human cytomegalovirus replication and on cytokine-mediated stimulation of endothelial cells. *Transplantation* **61**, 1763–1770.

6 Schreiber SL (1991) Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* **251**, 283–287.

7 Seizer P, Ochmann C, Schönberger T, Zach S, Rose M, Borst O, Klingel K, Kandolf R, MacDonald HR, Nowak RA *et al.* (2011) Disrupting the EMMPRIN (CD147)-cyclophilin A interaction reduces infarct size and preserves systolic function after myocardial ischemia and reperfusion. *Arterioscler Thromb Vasc Biol* **31**, 1377–1386.

8 Seko Y, Fujimura T, Taka H, Mineki R, Murayama K and Nagai R (2004) Hypoxia followed by reoxygenation induces secretion of cyclophilin A from cultured rat cardiac myocytes. *Biochem Biophys Res Commun* **317**, 162–168.

9 Zhang H, Chen J, Liu F, Gao C, Wang X, Zhao T, Liu J, Gao S, Zhao X, Ren H *et al.* (2014) CypA, a gene downstream of HIF-1alpha, promotes the development of PDAC. *PLoS One* **9**, e92824.

10 Sherry B, Yarlett N, Strupp A and Cerami A (1992) Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. *Proc Natl Acad Sci USA* **89**, 3511–3515.

11 Kim SH, Lessner SM, Sakurai Y and Galis ZS (2004) Cyclophilin A as a novel biphasic mediator of endothelial activation and dysfunction. *Am J Pathol* **164**, 1567–1574.

12 Suzuki J, Jin ZG, Meoli DF, Matoba T and Berk BC (2006) Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ Res* **98**, 811–817.

13 Braaten D and Luban J (2001) Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. *EMBO J* **20**, 1300–1309.

14 Luban J, Bossolt KL, Franke EK, Kalpana GV and Goff SP (1993) Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**, 1067–1078.

15 Luban J (1996) Absconding with the chaperone: essential cyclophilin-Gag interaction in HIV-1 virions. *Cell* **87**, 1157–1159.

16 Liu X, Sun L, Yu M, Wang Z, Xu C, Xue Q, Zhang K, Ye X, Kitamura Y and Liu W (2009) Cyclophilin A interacts with influenza A virus M1 protein and impairs the early stage of the viral replication. *Cell Microbiol* **11**, 730–741.

17 Zhou D, Mei Q, Li J and He H (2012) Cyclophilin A and viral infections. *Biochem Biophys Res Commun* **424**, 647–650.

18 Wang Y, Zhou X, Debing Y, Chen K, Van Der Laan LJ, Neyts J, Janssen HL, Metselaar HJ, Peppelenbosch MP and Pan Q (2014) Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology* **146**, 1775–1783.

19 Kawasaki H, Mocarski ES, Kosugi I and Tsutsui Y (2007) Cyclosporine inhibits mouse cytomegalovirus infection via a cyclophilin-dependent pathway specifically in neural stem/progenitor cells. *J Virol* **81**, 9013–9023.

20 Keyes LR, Bego MG, Soland M and St Jeor S (2012) Cyclophilin A is required for efficient human cytomegalovirus DNA replication and reactivation. *J Gen Virol* **93**, 722–732.

21 Qing J, Wang Y, Sun Y, Huang J, Yan W, Wang J, Su D, Ni C, Li J, Rao Z *et al.* (2014) Cyclophilin A associates with enterovirus-71 virus capsid and plays an essential role in viral infection as an uncoating regulator. *PLoS Pathog* **10**, e1004422.

22 Wen DQ, Zhang YY, Lv LP, Zhou XP, Yan F, Ma P and Xu JB (2009) Human cytomegalovirus-encoded chemokine receptor homolog US28 stimulates the major immediate early gene promoter/enhancer via the induction of CREB. *J Recept Signal Transduct Res* **29**, 266–273.

23 Stanton RJ, McSharry BP, Rickards CR, Wang EC, Tomasec P and Wilkinson GW (2007) Cytomegalovirus destruction of focal adhesions revealed in a high-throughput Western blot analysis of cellular protein expression. *J Virol* **81**, 7860–7872.

24 Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T and Schmid FX (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* **337**, 476–478.

25 Hopkins S and Gallay PA (2015) The role of immunophilins in viral infection. *Biochim Biophys Acta* **1850**, 2103–2110.

26 Saini M and Potash MJ (2006) Novel activities of cyclophilin A and cyclosporin A during HIV-1 infection of primary lymphocytes and macrophages. *J Immunol* **177**, 443–449.

27 Marks AR (1996) Cellular functions of immunophilins. *Physiol Rev* **76**, 631–649.

28 Galat A and Metcalfe SM (1995) Peptidylproline cis/trans isomerases. *Prog Biophys Mol Biol* **63**, 67–118.

29 Siekierka JJ, Hung SH, Poe M, Lin CS and Sigal NH (1989) A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**, 755–757.

30 Handschumacher RE, Harding MW, Rice J, Drugge RJ and Speicher DW (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226**, 544–547.

31 Cai J, Chen Y, Seth S, Furukawa S, Compans RW and Jones DP (2003) Inhibition of influenza infection by glutathione. *Free Radic Biol Med* **34**, 928–936.

32 Staal FJ, Roederer M and Herzenberg LA (1990) Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA* **87**, 9943–9947.

33 Paracha UZ, Fatima K, Alqahtani M, Chaudhary A, Abuzenadah A, Damanhouri G and Qadri I (2013) Oxidative stress and hepatitis C virus. *Virol J* **10**, 251.

34 Chatterji U, Bobardt M, Selvarajah S, Yang F, Tang H, Sakamoto N, Vuagniaux G, Parkinson T and Gallay P (2009) The isomerase active site of cyclophilin A is critical for hepatitis C virus replication. *J Biol Chem* **284**, 16998–17005.

35 An P, Wang LH, Hutcheson-Dilks H, Nelson G, Donfield S, Goedert JJ, Rinaldo CR, Buchbinder S, Kirk GD, O'Brien SJ *et al.* (2007) Regulatory polymorphisms in the cyclophilin A gene, PPIA, accelerate progression to AIDS. *PLoS Pathog* **3**, e88.

36 Hong F, Lee J, Song JW, Lee SJ, Ahn H, Cho JJ, Ha J and Kim SS (2002) Cyclosporin A blocks muscle differentiation by inducing oxidative stress and inhibiting the peptidyl-prolyl-cis-trans isomerase activity

of cyclophilin A: cyclophilin A protects myoblasts from cyclosporin A-induced cytotoxicity. *FASEB J* **16**, 1633–1635.

37 Tilton C, Clippinger AJ, Maguire T and Alwine JC (2011) Human cytomegalovirus induces multiple means to combat reactive oxygen species. *J Virol* **85**, 12585–12593.

38 van de Berg PJ, Heutinck KM, Raabe R, Minnee RC, Young SL, van Donselaar-van der Pant KA, Bemelman FJ, van Lier RA and ten Berge IJ (2010) Human cytomegalovirus induces systemic immune activation characterized by a type 1 cytokine signature. *J Infect Dis* **202**, 690–699.

39 Gui X, Ho M and Camp PE (1982) Effect of cyclosporin A on murine natural killer cells. *Infect Immun* **36**, 1123–1127.

40 Bharadwaj U, Zhang R, Yang H, Li M, Doan LX, Chen C and Yao Q (2005) Effects of cyclophilin A on myeloblastic cell line KG-1 derived dendritic like cells (DLC) through p38 MAP kinase activation. *J Surg Res* **127**, 29–38.

41 Kim H, Oh Y, Kim K, Jeong S, Chon S, Kim D, Jung MH, Pak YK, Ha J, Kang I *et al.* (2015) Cyclophilin A regulates JNK/p38-MAPK signaling through its physical interaction with ASK1. *Biochem Biophys Res Commun* **464**, 112–117.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Cell viability was quantitatively evaluated by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay.

Fig. S2. Silencing CyPA in HFF cells was evaluated using RT-qPCR and Western blotting to assess protein expression levels of CyPA in HFF mock, HFF siCONTROL (siCTR) and HFF siCyPA cells.

Fig. S3. The impact of cyclosporine A (CsA, 1 μM) on the expression levels of CyPA in HFF cells following H_2O_2 treatment (200 μM) was assessed by RT-qPCR and western blotting (A). Staining and the densitometric analysis of 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescence in the response of H_2O_2 and CsA (B).