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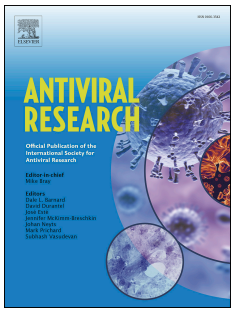

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Umifenovir effectively inhibits IL-10 dependent persistent Coxsackie B4 virus infection

Shilin Zhang[a], Chenyang Zhi[b], Hongrui Li[c], Dianshuai Huang[c], Qingjie Fan[c], Jiuwei Cui[a,*], Chongyang Liang[c,**]

[a] Cancer center, the First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, People's Republic of China

[b] Anus bowel division, Affiliated Hospital of Changchun University of Chinese Medicine, 1478 Gongnong Road, Changchun 130021, People's Republic of China

[c] Institute of Frontier Medical Science of Jilin University, 1163 Xinmin Street, Changchun 130021, People's Republic of China

[*]Corresponding author. Tel.: +86 431 88782178; fax: +86 431 88782178.

[**]Corresponding author. Tel.: +86 431 85619387; fax: +86 431 85622960.

E-mail address: cuijw@jlu.edu.cn (J. Cui), liang@jlu.edu.cn (C. Liang)

Abstract

Coxsackie virus cannot be completely eliminated due to restrictive replication and impaired immune response, thus causing persistent infection. IL-10 plays a decisive role in the course of persistent viral infection. Umifenovir is a broad-spectrum antiviral drug, with certain treatment effects on Coxsackie virus infection. Previously, we showed that in addition to inhibiting Coxsackie B4 (CVB4) infection, Umifenovir also down-regulates IL-10 induced by persistent CVB4 virus infection *in vitro* and *in vivo*. Here, BALB/c mouse spleen cells infected with CVB4 were used as a model to explore the mechanism by which Umifenovir affects IL-10 expression. We found that subcellular localization of p38 and MAPK-activated protein kinase 2 (MK2) played a very important role in IL-10 secretion, and Umifenovir significantly prevented p38-MK2 complex from exiting the cell nucleus. This in turn blocked the biological functions of the latter pathway, and inhibited the high expression of IL-10 induced by CVB4. These findings suggest that Umifenovir is a potential anti-CVB4 drug; most importantly, Umifenovir could be used to treat IL-10 induced persistent viral infection.

Keywords: Umifenovir; Coxsackie B4; viral myocarditis; IL-10; P38 MAPK; MAPK-activated protein kinase 2

Abbreviations

CVB4, Coxsackie B4; MK2, MAPK-activated protein kinase 2; APC, Antigen presenting cells; LCMV, Lymphocytic choriomeningitis virus; HCV, Hepatitis C virus; CMV, Cytomegalovirus; EBV, Epstein-Barr virus; ERK, Extracellular-signal regulated protein kinase; TLR2, Toll-like receptor 2; PRRSV, Porcine reproductive and respiratory syndrome virus

1. Introduction

Antigen presenting cells (APC) present viral antigens to T cells after viral infection, guide virus specific T cell proliferation mediated by soluble cytokines, and exert antiviral and immunological stress effects, thus eliminating viral infection (Brooks et al., 2010). Suppressing the immune response could impair the management of viral replication, thus inducing persistent infection. Persistent viral infection is one of the major issues threatening human health; IL-10 has immune suppressive effects and could persistently inhibit T cell activity, terminate T cell response, and directly influence co-stimulatory molecules of APC to prevent T cell activation (Brooks et al., 2010). Therefore, IL-10 is considered a critical factor in persistent viral infection (Shuang et al., 2013). Brooks et al. (2006) showed that persistent infection by lymphocytic choriomeningitis virus (LCMV) increases IL-10 expression and damages T cell response, thus inducing persistent viral infection. IL-10 knockdown could help restore normal T cell response, rapidly eliminating the virus and initiating an anti-viral response by memory T cells. Animal studies showed that supplying IL-10 specific antibody to block IL-10 receptor could restore T cell function and eliminate viral infection (Brooks et al., 2006).

Umifenovir is a broad-spectrum anti-viral drug (Blaising et al., 2014) widely used in clinical practice. Eve-Isabelle et al. (2007) suggested that Umifenovir binds to the plasma membrane and inhibits hepatitis C virus (HCV) envelope fusion to the host cell membrane, thus blocking viral replication. Meanwhile, Leneva et al. (2009) found that Umifenovir could stabilize hemagglutinin and further inhibit viral fusion with host cells, therefore preventing viral replication. Due to issues related to Umifenovir development, studies assessing the immunological mechanisms of Umifenovir are scarce. We found that in addition to its anti-CVB4 viral effects, Umifenovir significantly affects T cell related

IL-10 secretion in our preliminary work. Therefore, we hypothesized that Umifenovir could inhibit IL-10 expression, thus inhibiting persistent infection of CVB4. The current findings provide a new notion for the treatment of IL-10 induced persistent viral infection, indicating that Umifenovir could be a potential drug to treat IL-10 induced persistent viral infection.

2. Materials and methods

2.1 Cells, viruses, and mouse strains

The standard CVB4 strain was propagated in Hela cells, which were cultured in DMEM containing 10% fetal bovine serum (FBS) until a TCID50 of 106.15/100μl (Reed and Muench, 1938). BALB/c mice of 5-6 weeks (15-18 g) were from the Academy of Military Medical sciences in Beijing, and raised in a SPF environment.

2.2 Drugs and reagents

Umifenovir (No. 110915; Qianjiang Hengshuo Chemical Industry Co. Ltd., Hubei) was the investigational drug. For cytological experiments, Umifenovir was dissolved in DMSO, whose volume was less than 0.05% to avoid inhibitory effects on viral replication (Shi et al., 2007). The other reagents used in this study were: p38MAPK inhibitor (SB203580, Sigma), Hoechst33342 (Sigma), anti-STAT3/p-STAT3 (Cell Signaling), anti-p38/p-p38 (SANTA CRUZ), anti-MK2/p-MK2 (Abcam), ELISA kits for IL-10 (BOSTER, EK0417), fluorescent quantitative PCR kit (TAKARA), Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies), Rabbit anti-mouse IgM-FITC (SANTA CRUZ) and Alexa Fluor 568 donkey anti-rabbit IgG (Life Technologies). PCR primers for CVB4 virus (Brilot et al., 2002), GADPH (Peng et al., 2016), and IL-10 (Bliss et al., 2003) were synthesized by Sangon biological

engineering co., LTD., Shanghai.

2.3 Antiviral activity of Umifenovir *in vitro* and *in vivo*

Myocardial cells collected from heart tissues of newborn BALB/c mice (1-2 days old) (Harary and Farley, 1960) were cultured (4×10^6 cells/ml) with CVB4 (1.4×10^6TCID50) virus solution; different doses of Umifenovir (2, 4, and 8 μg/ml) were added at seeding. In the normal- and virus- control groups, the same volume of culture medium was added. IL-10 levels in supernatants were measured by ELISA, and CVB4 viral load in cell lysates was assessed by real-time RT-PCR.

Thirty male BALB/c mice were randomly divided into 5 groups (n=6) and injected intraperitoneally with 0.2 ml CVB4 (2.8×10^3TCID50) or saline in the normal control group for 3 days to establish a myocarditis model. Virus inoculated mice were then treated with Umifenovir at 100, 200, and 300 $mg\cdot kg^{-1}\cdot d^{-1}$, and saline, respectively. The drugs were administered in 0.2 ml solution by gavage, twice a day from the day before infection for 8 days. The mice were sacrificed 7 days after infection, and serum was collected to assess IL-10 levels by ELISA. Heart tissue specimens were employed for RNA extraction (Chomczynski and Sacchi, 1987); reverse transcription and real-time SYBR fluorescent quantitative PCR were performed to measure CVB4 viral load and IL-10 mRNA levels. The remaining tissue samples were fixed in 10% formaldehyde for H&E staining.

2.4 Protein quantitation by ELISA and western blot

Spleen cells were obtained from BALB/c mice as previously described (Yoshida et al., 1997), and infected with CVB4 (1.4×10^6TCID50), adding different doses of Umifenovir (2, 4, and 8 μg/ml).and Rupintrivir (5, 10, 20 and 40μM) respectively. In the normal- and virus- control groups, the same volume of culture medium was added. Supernatants were collected 24 h later, and the level of IL-10

was measured by ELISA. Meanwhile, total protein was extracted from the cells, and protein expression levels of STAT3/p-STAT3, p38/p-p38 and MK2/p-MK2 were measured with the Wes automatic western blot analysis system (Wes, Protein simple).

2.5 Effect of p38MAPK inhibitor on IL-10 secretion

Spleen cells were obtained from BALB/c mice, with the p38 MAPK inhibitor SB203580 added while infecting the cells with CVB4 virus (1.4×10^6TCID50). Final concentrations of SB203580 were 2.5, 5, 10, 20, and 40 μM; a virus control group was also included. IL-10 protein levels in supernatants were measured by ELISA after 48 h of incubation.

Hela cells were seeded in 96-well at 1×10^4 cells/well, infected with CVB4 (2.8×10^3TCID50) and treated with SB203580 (0μM, 5μM, 10μM, 20μM, 40μM, 80μM) the following day. After 48h incubation, the viability was measured in WST-1.

2.6 Cellular localization of p38 and MK2 by 3D-SIM super-resolution imaging

Spleen cells were extracted from BALB/c mice. After infection with CVB4 (1.4×10^6TCID50) virus, different doses of Umifenovir (2, 4, and 8 μg/ml) and Rupintrivir (5, 10, 20 μM) were added respectively; the same volume of culture medium was added to normal- and virus- control groups. The cells were collected after 24 h of incubation, fixed, and sequentially incubated with primary antibodies (against p38/p-p38 and MK2/p-MK2 at 1:500) and fluorescent secondary antibodies (1:1000). A super resolution microscope (DeltaVision OMX, OM06051, GE Healthcare) was used for analyses.

3. Results

3.1 Umifenovir inhibits CVB4 infection *in vitro* and *in vivo*

To assess the antiviral activity of Umifenovir, CVB4 virus was inoculated into myocardial cells of newborn mice, and different doses of Umifenovir (2, 4, and 8 μg/ml) were added for treatment. Interestingly, Umifenovir significantly inhibited the viral load in a dose-dependent manner ($p<0.01$) (Fig. 1A). To further verify the anti-CVB4 effects of Umifenovir, CVB4 virus amounts in myocardial tissues from the mouse models of viral myocarditis were assessed after treatment with Umifenovir (100, 200, and 300 $mg \cdot kg^{-1} \cdot d^{-1}$) (Fig. 1D). The results corroborated *in vitro* findings. Indeed, Umifenovir significantly reduced the viral load in myocardial tissues, compared with the virus control group ($p<0.01$). In addition, pathological sections in Fig.1G showed large areas of infarction with inflammatory cell infiltration in the virus model group. These infarction areas decreased markedly with increasing Umifenovir doses (Fig. 1H, I, J), suggesting that Umifenovir had protective effects on myocardial damage induced by CVB4 virus.

3.2 Umifenovir inhibits CVB4 infection induced IL-10 over-expression

Schmidtke et al. (2000) reported abnormally increased IL-10 expression in chronic myocarditis models induced by Coxsackie virus. Brooks et al. (2010) found that increased IL-10 expression induces persistent viral infection. Therefore, we used the above models to assess whether Umifenovir could also down-regulate IL-10 in addition to its anti-CVB4 virus activity. After infection with CVB4 virus for 24 or 48 h, large amounts of IL-10 were secreted from newborn mouse myocardial cells (Fig. 1B); however, when different doses of Umifenovir were added, IL-10 levels were reduced significantly ($p<0.01$). In viral myocarditis assays (Fig. 1E), CVB4 viral infection

significantly stimulated the abnormal expression of IL-10 in myocardial tissues, while 100, 200, and 300 mg/kg of Umifenovir dose-dependently inhibited CVB4 virus infection related IL-10 increase ($p<0.01$), in agreement with *in vitro* findings. After infection with the virus, IL-10 levels in serum samples were 11 fold the amounts in normal mice (Fig. 1C); after treatment with Umifenovir, the inhibitory effects of Umifenovir increased in a dose-dependent manner ($p<0.01$). These findings demonstrated that CVB4 virus infection induced IL-10 expression, while Umifenovir significantly inhibited IL-10 expression in addition to its anti-viral effects.

3.3 IL-10 secretion can be inhibited by Umifenovir directly

In most studies, LPS is often used to stimulate IL-10 secretion. We preliminary evaluated the effect of Umifenovir on IL-10 secretion stimulated by LPS, and it showed that Umifenovir could significantly inhibit IL-10 secretion in a dose-dependent manner. (Fig. S2).

CVB4 infection could induce the abnormal secretion of IL-10 too. Spleen cells from BALB/c mice infected with CVB4 were used to assess the effects of Umifenovir on the secretion of IL-10. Concentrations of IL-10 in the virus control group averaged 2701.52 pg/ml, significantly higher compared with the normal control group (87.70 pg/ml). Umifenovir could significantly reduce IL-10 secretion induced by CVB4 infection, with 89.19 pg/ml IL-10 obtained at 8 μg/ml, a value very close to that of the normal group. Data about the concentration of IL-10 was showed in Fig. 2G.

In addition, we selected Rupintrivir, another antiviral drug used for CVB4 infection as the control of Umifenovir. We also found that CVB4 infection could promote IL-10 secretion, but Rupintrivir could not inhibit the high expression of IL-10 (Fig. S3 and Fig. 2H). That is to say, Rupintrivir has antiviral effect on CVB4 infection only, and has no effect on IL-10 secretion which is the key of persistent

infection. This result is different from Umifenovir, which could inhibit CVB4 infection directly and control CVB4 persistent infection induced by IL-10 secretion.

3.4 Phosphorylation of p38MAPK and MK2 mediates IL-10 downregulation by Umifenovir

To further explore the mechanisms underlying the inhibitory effects of Umifenovir on IL-10 expression, we assessed the pathways related to IL-10 expression, including p38-MAPK and STAT3 (Dáňová et al., 2015; Mion et al., 2014). As shown in Fig. 2, CVB4 virus infection reduced p38 phosphorylation, while phosphorylation of the downstream MK2 was increased. However, treatment with Umifenovir significantly promoted p38 phosphorylation and reduced MK2 phosphorylation. Therefore, we speculated that CVB4 virus infection induced IL-10 expression was mainly through the p38MAPK pathway. In order to verify this view, SB203580 was used to inhibit p38MAPK signal transduction (Fig. 2E), and changes of IL-10 expression after CVB4 virus infection were measured. We also evaluated the antiviral effect of SB203580 on CVB4 infection and it showed that SB203580 could inhibit p38MAPK signal transduction and IL-10 secretion when the concentration was 0.6 uM according to the instruction. But it had no antiviral against to CVB4 infection at this concentration. And SB203580 could inhibit CVB4 infection significantly only when the concentration reached 40 μM which is far more than the working concentration (0.6 μM). (Fig. 2F)

3.5 Umifenovir causes nuclear export disruption of the p38-MK2 complex

Phosphorylation of the p38 and MK2 proteins plays extremely important roles in the inhibitory effects of Umifenovir on IL-10 expression induced by CVB4. MK2 is a distant kinase of the p38MAPK signaling pathway cascade and phosphorylation substrate of p38. MK2 phosphorylation by p38 occurs in the cell nucleus, and p38 translocation into the nucleus only occurs after p38

phosphorylation by MAP2Ks in the cytoplasm, which is not associated with the catalytic activity of the kinase (Cuadrado and Nebreda, 2010; ter Haar et al., 2007). Activated p38 in the nucleus binds and activates MK2 at its phosphorylation sites, then the p38-MK2 complex is exported to the cytoplasm to phosphorylate its substrates (Ben-Levy et al., 1998; Gong et al., 2010). So p38 translocation plays an important role in the p38MAPK-MK2 pathway and when blocked, p38MAPK-MK2 is also blunted, thus inhibiting IL-10 expression.

Therefore, the 3D-SIM imaging system was used to scan the cells layer by layer, after which image reconstruction was performed to assess cell distribution of p-p38 and p-MK2. The results showed that p-p38 signals were mainly distributed in the cytoplasm (green fluorescence in Fig. 3) while p-MK2 signals are mainly found in the nucleus (green fluorescence in Fig. 4). After viral infection, p-p38 percentage in the nucleus was significantly reduced, while cytoplasmic p-p38 was increased significantly. In addition, p-MK2 amounts were significantly reduced in the nucleus. However, with increasing Umifenovir levels, both p-p38 and p-MK2 contents in the cell nucleus increased correspondingly, with significant differences obtained compared with the virus control group. After treatment with Umifenovir, p-p38 translocated into the nucleus and phosphorylated MK2, but could not translocate from the nucleus to cytoplasm. These findings indicated that Umifenovir prevented the nuclear export of p38-MK2 to block the p38MAPK-MK2 signaling pathway, subsequently inhibiting IL-10 expression. The levels of p-MK2 were not consistent with Western blot data. In Western blot assays, complete lysis-M (EDTA-free), a moderate cell lysis buffer, was used, which was not powerful enough to lyse cell nuclei; therefore, p-MK2 levels obtained by Western blot could only reflect cytoplasmic p-MK2 levels. Thus, discrepant results were obtained in Western blot and 3-D imaging. However, the similar situations were not observed in the positive control that was

treated with Rupintrivir. Particularly, the distribution of p-p38 and p-MK2 intranuclear had no significant difference with the viral control group after treated with Rupintrivir (Fig. 5 and Fig.6). The found indicated that Rupintrivir had no inhibitory effect on the nuclear export of p38-MK2 like Umifenovir and the p38MAPK-MK2 pathway could not be blocked by Rupintrivir. So Rupintrivir could not inhibit IL-10 secretion and this result was consistent with the result in ELISA. We concluded that although Rupintrivir has antiviral activity, it cannot inhibit IL-10 secretion to control persistent viral infection, however Umifenovir has the dual-function above.

4. Statistical Analysis

Significant differences in the mean amplitude and frequency were tested using the Student paired 2-tailed t test. Values of $p<0.05$ were considered significant. And we used GraphPad Prism5 software (GraphPad Software, Inc., La Jolla, CA) for statistical analysis.

5. Discussion

Virus elimination relies on functional T cell response; however, T cells may lose replication capability when infected with certain viruses, and therefore could not lyse the infected cells. The lack of T cell response could directly mean failed virus eradication, promoting persistent viral infection (Brooks et al., 2008). Persistent viral infection is a major issue that severely threatens human health. Its occurrence is closely associated with the immune system, with cytokines produced by macrophages and T lymphocytes playing critical roles in anti-viral immune responses. These cytokines modulate the expression of MHC-, adhesion-, and co-stimulatory molecules, and directly activate or deactivate immune cells; such changes stimulate anti-viral responses by NK and CTL

cells, and regulate the antibody-mediated virus eradication (Ramshaw et al., 1997). Recent studies found that in addition to inhibiting and eliminating viruses, some antiviral drugs also have immune modulating functions. For instance, Entecavir (ETV) and Adefovir (ADV) do not only effectively inhibit HBV replication, but also promote the secretion of Th1 cytokines, including IL-2, IFN-γ, and TNF-α (Jiang et al., 2011). Acyclovir, a drug mainly used for treating herpes virus infection, also inhibits IL-12 and IL-27 secretion (Alessandra et al., 2014).

The present study assessed whether Umifenovir could also affect the expression of multiple cytokines while inhibiting CVB4 virus infection. As shown above, Umifenovir significantly inhibited the expression of IL-10, a cytokine closely associated with persistent viral infection. IL-10 is a critical factor in persistent viral infection. Brooks et al. (2008) found that using anti-IL-10 antibody to block IL-10 during persistent viral infection improves the T cell function, thus reducing virus titers. Wilson and Brooks et al. (2011) also demonstrated that when IL-10 activity is neutralized, e.g. in IL-10 gene knockout mice or by using antibodies to block IL-10, immune functions could be continued, with persistent viral infection rapidly eliminated. In accordance with previous findings, the present study confirmed that infection by CVB4 virus both *in vivo* and *in vitro* induces an abnormal secretion of IL-10. However, this abnormal secretion was inhibited by appropriate doses of Umifenovir, suggesting that Umifenovir effectively inhibited IL-10 expression while exerting anti-CVB4 virus activity.

During persistent viral infection, activation of multiple immune signals induces IL-10 expression. For instance, HCV, CMV, EBV, and LCMV bind to Toll-like receptor 2 (TLR2), recruit MyD88, and activate the extracellular-signal regulated protein kinase (ERK) signaling pathway to induce IL-10 expression (Ariza et al., 2009; Zhou et al., 2008). After infection with porcine reproductive and

respiratory syndrome virus (PRRSV), macrophages induce IL-10 expression via p38MAPK and NF-κB pathways (Hou et al., 2012). In patients with HIV infection, the HIV glycoprotein binds to mannose C-type lectin receptors (such as DC-SIGN) on the surface of monocyte-derived DC, inducing IL-10 expression (Meimei et al., 2007; Wilson and Brooks, 2011). Maher et al.(2014) also showed that macrophages secrete IL-10 through the STAT3 dependent pathway. Ehlting et al. (2016) showed that during cytomegalovirus infection, MK2, a kinase downstream of p38, regulates IL-10 expression and prevents aggregation of bone marrow cells in the liver. These findings suggest that the mechanisms involved in IL-10 induction by different viruses are distinct; this could result from inherent differences in pathogen-specific responses. The present study demonstrated that CVB4 virus infection induced changes in the expression of p38MAPK signaling effectors that mediate IL-10 secretion, among which p38 and MK2 phosphorylation was most significant.

Phosphorylation of p38 and MK2 is closely associated with the nuclear translocation of p38. In the resting status, p38 is mainly distributed in the cytoplasm, while stress induces a conformational change of p38, which can then recognize the nuclear localization signal (NLS)-containing protein and enters the nucleus (Cuadrado and Nebreda, 2010; Gong et al., 2010). Previous studies. demonstrated that p38α has no nuclear export signal, and only binding to MK2a and forming a dimer in the nucleus would allow it to exit the nucleus. Briefly, p38α is phosphorylated by MKK3 and MKK6, while activated p38α binds to the phosphorylation site and activates MK2a. The activated MK2a undergoes a conformational change and exposes the nuclear export signal. Activated p38α and MK2 then translocate from nucleus to cytoplasm, and further phosphorylate the cytoplasmic substrates (Ben-Levy et al., 1998; ter Haar et al., 2007). Therefore, the effects of Umifenovir on IL-10 are associated with MK2 phosphorylation and nuclear translocation of the p38-MK2 complex. We used

the 3D-SIM imaging system to assess the localization of p38 and MK2, two proteins of the p38MAPK-MK2 pathway in spleen cells. Interestingly, CVB4 virus infection stimulated p38-MK2 translocation from the nucleus, as p-p38 and p-MK2 percentages in the nucleus were significantly lower in the virus groups compared with the normal control group. Meanwhile, Umifenovir inhibited the nuclear export process of p38-MK2, thus blocking the p38MAPK-MK2 signaling pathway, inhibiting subsequent biological functions, and finally altering IL-10 secretion.

Spleen cells are about 5-10μm in size, and it is relatively difficult to use confocal microscopy to observe the dynamic changes of subcellular localization of the functional complex in the nucleus and cytoplasm. Therefore, we used 3D-SIM Super-resolution Microscopy and the iMaris 3D rebuilding software for combined analysis of p38/p-p38 and MK2/p-MK2 localization in spleen cells. This method has higher resolution for analyzing the subcellular localization of protein complexes, and provides intuitive results as well.

In summary, the present study assessed the mechanisms by which Umifenovir inhibits CVB4 infection persistence, and showed that Umifenovir affected p-p38 and p-MK2 localization and inhibited the nuclear export of p38-MK2, thus inhibiting IL-10 expression.

As the nuclear export of p38-MK2 is too fast to detect, the instrument could not capture the complex going through the nuclear membrane. Because of the competitive relationship among antibodies, we were not able to label p38 and MK2 with different antibodies to detect co-localization at the same time. These problems will be addressed in future studies.

Umifenovir is a commercially available antiviral drug with stable structure, and broad-spectrum antiviral effects. The present study also demonstrated that Umifenovir could effectively treat

persistent viral infection. Umifenovir inhibited the nuclear export of p38-MK2 to block the p38MAPK-MK2 signaling pathway; this reduces IL-10 expression, antagonizes IL-10 induced immune suppressive effects, and promotes the immune system to reduce viral infection persistence. Therefore, Umifenovir should be used not only as an anti-viral agent, but also for treating persistent viral infection and immune suppressive diseases, against which this drug may also exert ideal effects.

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References

Alessandra, F., Giuditta Fiorella, S., Maria Filomena, P., Linda, P., Mauro, M., Giorgio, B., 2014. Effect of the N-butanoyl glutathione (GSH) derivative and acyclovir on HSV-1 replication and Th1 cytokine expression in human macrophages. Medical Microbiology & Immunology 203, 283-289.

Ariza, M., Glaser, R., Pt, Jones, C., Williams, M., 2009. The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. Journal of Immunology 182, 851-859.

Ben-Levy, R., Hooper, S., Wilson, R., Paterson, H.F., Marshall, C.J., 1998. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. Current biology : CB 8, 1049-1057.

Blaising, J., Polyak, S.J., Pécheur, E.-I., 2014. Arbidol as a broad-spectrum antiviral: An update. Antiviral Research 107,

84-94.

Bliss, S.K., Alcaraz, A., Appleton, J.A., 2003. IL-10 prevents liver necrosis during murine infection with Trichinella spiralis. J Immunol 171, 3142-3147.

Brilot, F., Chehadeh, W., Charlet-Renard, C., Martens, H., Geenen, V., Hober, D., 2002. Persistent infection of human thymic epithelial cells by coxsackievirus B4. J Virol 76, 5260-5265.

Brooks, D.G., Sang-Jun, H., Heidi, E., Sharpe, A.H., Freeman, G.J., Oldstone, M.B.A., 2008. IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. Proceedings of the National Academy of Sciences 105, 20428-20433.

Brooks, D.G., Trifilo, M.J., Edelmann, K.H., Teyton, L., McGavern, D.B., Oldstone, M.B., 2006. Interleukin-10 determines viral clearance or persistence in vivo. Nat Med 12, 1301-1309.

Brooks, D.G., Walsh, K.B., Heidi, E., Oldstone, M.B.A., 2010. IL-10 directly suppresses CD4 but not CD8 T cell effector and memory responses following acute viral infection. Proceedings of the National Academy of Sciences 107, 3018-3023.

Chomczynski, P., ., Sacchi, N., . 1987. Single step method of RNA isolation by Acid Guanidium Thiocyanate-Phenol-Chloroform Extraction. Analytical Biochemistry 162, 156-159.

Cuadrado, A., Nebreda, A.R., 2010. Mechanisms and functions of p38 MAPK signalling. The Biochemical journal 429, 403-417.

Dáňová, K., Klapetková, A., Kayserová, J., Šedivá, A., Špíšek, R., Jelínková, L.P., 2015. NF-κB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/dexamethasonegenerated tolerogenic dendritic cells in the inflammatory environment. Oncotarget 6, 14123-14138.

Ehlting, C., Trilling, M., Tiedje, C., Le-Trilling, V.T., Albrecht, U., Kluge, S., Zimmermann, A., Graf, D., Gaestel, M., Hengel, H., Haussinger, D., Bode, J.G., 2016. MAPKAP kinase 2 regulates IL-10 expression and prevents formation of intrahepatic

myeloid cell aggregates during cytomegalovirus infections. J Hepatol 64, 380-389.

Estcourt, M.J., Ramshaw l, A., Ramsay, A.J., 1998. Cytokine responses in virus infections: effects on pathogenesis, recovery and persistence. Curr Opin Microbiol 1, 411-418.

Eve-Isabelle, P., Dimitri, L., Fanny, A., Jennifer, M., Boriskin, Y.S., Michael, R., Cosset, F.o.-L.c., Polyak, S.J., 2007. Biochemical mechanism of hepatitis C virus inhibition by the broad-spectrum antiviral arbidol. Biochemistry 46, 6050-6059.

Gong, X., Ming, X., Deng, P., Jiang, Y., 2010. Mechanisms regulating the nuclear translocation of p38 MAP kinase. Journal of cellular biochemistry 110, 1420-1429.

Harary, I., Farley, B., 1960. In vitro studies of single isolated beating heart cells. Science 131, 1674-1675.

Harker, J.A., Dolgoter, A., Zuniga, E.I., 2013. Cell-intrinsic IL-27 and gp130 cytokine receptor signaling regulates virus-specific CD4(+) T cell responses and viral control during chronic infection. Immunity 39, 548-559.

Hou, J., Wang, L., Quan, R., Fu, Y., Zhang, H., Feng, W.H., 2012. Induction of interleukin-10 is dependent on p38 mitogen-activated protein kinase pathway in macrophages infected with porcine reproductive and respiratory syndrome virus. Virology Journal 9, 1733-1733.

Jiang, Y., Li, W., Yu, L., Liu, J., Xin, G., Yan, H., Sun, P., Zhang, H., Xu, D., Niu, J., 2011. Enhancing the antihepatitis B virus immune response by adefovir dipivoxil and entecavir therapies. Cell Mol Immunol 8, 75-82.

Kumar, R.M., Anbazhagan, R., Satheesh, C.C., Balamurugan, V., Mrudula, V., Porteen, K., 2006. Role of Viral Cytokines (Virokines) in Viral Infections-Mini Review. International Journal of Molecular Medicine & Advance Sciences, 121-127.

Leneva, I.A., Russell, R.J., Boriskin, Y.S., Hay, A.J., 2009. Characteristics of arbidol-resistant mutants of influenza virus: implications for the mechanism of anti-influenza action of arbidol. Antiviral Res 81, 132-140.

Maher, K., Zavr, J., #x, nik, Jeri, B., d, x., -Kokelj, Vasiljeva, O., Turk, B., Nata, #x, Kopitar-Jerala, a., 2014. Decreased IL-10

expression in stefin B-deficient macrophages is regulated by the MAP kinase and STAT-3 signaling pathways. Febs Letters 588, 720-726.

Mauro, E., Gattei, V., Mazzaro, C., 2014. Recombinant Human Erythropoietin (RHuEpo) and Granular Colony Stimulating Factor (G-CSF) in hepatitis C virus (HCV) related to mixed cryoglobulinaemia associated to membranoproliferative glomerulonephritis type I: a case report description. Le infezioni in medicina : rivista periodica di eziologia, epidemiologia, diagnostica, clinica e terapia delle patologie infettive 22, 337-341.

Meimei, S., Per Johan, K., Kaustuv, B., Dey, A.K., Iyer, S.P.N., Robert, D., Dustin, C., Lila, C.G., Olson, W.C., Sanders, R.W., 2007. HIV-1 gp120 mannoses induce immunosuppressive responses from dendritic cells. Plos Pathogens 3, e169-e169.

Mion, F., Tonon, S., Toffoletto, B., Cesselli, D., Pucillo, C.E., Vitale, G., 2014. IL-10 production by B cells is differentially regulated by immune-mediated and infectious stimuli and requires p38 activation. Molecular Immunology 62, 266–276.

Montaner, L.J., Doyle, A.G., Collin, M., Herbein, G., Illei, P., James, W., Minty, A., Caput, D., Ferrara, P., Gordon, S., 1993. Interleukin 13 inhibits human immunodeficiency virus type 1 production in primary blood-derived human macrophages in vitro. The Journal of experimental medicine 178, 743-747.

Peng, S., Sheng, W., Xia, W., Wang, J.Q., Zhang, Y.Y., Qiao, M.Q., Wu, J.B., 2016. Anger Emotional Stress Influences VEGF/VEGFR2 and Its Induced PI3K/AKT/mTOR Signaling Pathway. Neural Plasticity 2016, 1-12.

Perno, C.F., Yarchoan, R., Cooney, D.A., Hartman, N.R., Webb, D.S., Hao, Z., Mitsuya, H., Johns, D.G., Broder, S., 1989. Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'3'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. The Journal of experimental medicine 169, 933-951.

Ramshaw, I.A., Ramsay, A.J., Karupiah, G., Rolph, M.S., Mahalingam, S., Ruby, J.C., 1997. Cytokines and immunity to viral infections. Immunol Rev 159, 119-135.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. American Journal of Tropical

Medicine & Hygiene 27.

Schmidtke, M., ck, B.G., Merkle, I., Hofmann, P., Stelzner, A., Gemsa, D., 2000. Cytokine profiles in heart, spleen, and thymus during the acute stage of experimental coxsackievirus B3-induced chronic myocarditis. Journal of Medical Virology 61, 518-526.

Shi, L., Xiong, H., He, J., Deng, H., Li, Q., Zhong, Q., Hou, W., Cheng, L., Xiao, H., Yang, Z., 2007. Antiviral activity of arbidol against influenza A virus, respiratory syncytial virus, rhinovirus, coxsackie virus and adenovirus in vitro and in vivo. Arch Virol 152, 1447-1455.

Shuang, S., Jing, B., Dang, W., Fang, L., Zhang, L., Feng, L., Chen, H., Xiao, S., 2013. Porcine reproductive and respiratory syndrome virus infection activates IL-10 production through NF-κB and p38 MAPK pathways in porcine alveolar macrophages. Developmental & Comparative Immunology 39, 265-272.

ter Haar, E., Prabhakar, P., Liu, X., Lepre, C., 2007. Crystal structure of the p38 alpha-MAPKAP kinase 2 heterodimer. The Journal of biological chemistry 282, 9733-9739.

Van, R.K., Van, G.S., Pensaert, M., 2002. Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs. Viral Immunology 15, 583-594.

Wilson, E.B., Brooks, D.G., 2011. The role of IL-10 in regulating immunity to persistent viral infections. Current topics in microbiology and immunology 350, 39-65.

Yoshida, Y., Wang, M.Q., Liu, J.N., Shan, B.E., Yamashita, U., 1997. Immunomodulating activity of Chinese medicinal herbs and Oldenlandia diffusa in particular. Int J Immunopharmacol 19, 359-370.

Zhou, S., Halle, A., Kurt-Jones, E.A., Cerny, A.M., Porpiglia, E., Rogers, M., Golenbock, D.T., Finberg, R.W., 2008. Lymphocytic choriomeningitis virus (LCMV) infection of CNS glial cells results in TLR2-MyD88/Mal-dependent inflammatory responses. J Neuroimmunol 194, 70-82.

Figure captions

Fig. 1 Effects of Umifenovir *in vitro* (2,4 and 8 μg/ml) and *in vivo* (100, 200 and 300 mg/kg) on anti-CVB4 virus infection and IL-10 secretion. (A) Relative expression measurement of CVB4 mRNA in cultured myocardial cells by RT-PCR. (B) IL-10 levels in cultured myocardial cell supernatants after treatment with Umifenovir for 24 or 48 h *in vitro*, as evaluated by ELISA. (C) ELISA showing serum IL-10 levels in mouse models of viral myocarditis. (D) Relative expression measurement of CVB4 mRNA in myocardial tissues from mouse models of viral myocarditis by RT-PCR. (E) Quantitative measurement by RT-PCR of IL-10 mRNA levels in myocardial tissues from mouse models of viral myocarditis.

*p<0.05, ** p<0.01.

F-J, H&E staining of pathological sections from myocardial tissues of mice with viral myocarditis. F, normal control group; G, virus control group; H, 100 $mg \cdot kg^{-1} \cdot d^{-1}$ Umifenovir treatment group; I, 200 $mg \cdot kg^{-1} \cdot d^{-1}$ Umifenovir treatment group; and J, 300 $mg \cdot kg^{-1} \cdot d^{-1}$ Umifenovir treatment group. Black arrows indicate infarction areas (×200).

Fig. 2 Umifenovir inhibits the p38MAPK signaling pathway related to IL-10 expression. (A) mouse spleen cells infected with CVB4 were treated with different doses of Umifenovir (2,4 and 8 μg/ml) for 24 h; Western blot was used to measure the levels of proteins of IL-10 related pathways (p-p38/p38, p-MK2/MK2 and p-STAT3/STAT3); (B) p-MK2 and MK2 density of each band from (A) was measured and compared to the normal control group. (C) p-p38 and p38 density of each band

from (A) was measured and compared to the normal control group. (D) p-STAT3 and STAT3 density of each band from (A) was measured and compared to the normal control group. (E) Mouse spleen cells infected with CVB4 were treated with the p38MAPK pathway inhibitor SB203580 for 48 h, and ELISA was used to measure IL-10 expression in supernatants. (F) Hela cells infected with CVB4 were treated with the p38MAPK pathway inhibitor SB203580 for 48 h, and the cell viability was measured by WST-1. (G) Mouse spleen cells infected with CVB4 were treated with Umifenovir for 48 h, and ELISA was used to measure IL-10 expression in supernatant. (H) Mouse spleen cells infected with CVB4 were treated with Rupintrivir for 48 h, and ELISA was used to measure IL-10 expression in supernatant.

Fig. 3 Analysis of p-p38 localization in spleen cells after CVB4 infection, treated with Umifenovir for 24 h. Hoechst 33342 was used for nuclear labeling (blue fluorescence). Rabbit anti-mouse IgM-FITC secondary antibodies were used for p-p38 detection (green fluorescence). (A) 3D-SIM imaging system (DeltaVision OMX) was used for analysis (Bars: 10μm): most p-p38 was distributed in the cytoplasm. (B) Reconstruction of p-p38 images from (A) confirmed p-p38 was mainly located in the cytoplasm. (Bars: 2μm). (C) Representative histograms are shown for p-p38 percentage in the nucleus.

Fig. 4 Analysis of p-MK2 localization in spleen cells after CVB4 infection, treated with Umifenovir for 24 h. Hoechst 33342 was used for nuclear labeling (blue fluorescence). Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies were used for p-MK2 detection (green

fluorescence). (A) 3D-SIM imaging system (DeltaVision OMX) was used for analysis (Bars: 10μm): most p-MK2 was distributed in the nucleus. (B) Reconstruction of p-MK2 images from (A) confirmed p-MK2 was mainly located in the nucleus (Bars: 2μm). (C) Representative histograms are shown for p-MK2 percentage in the nucleus.

Fig. 5 Analysis of p-p38 localization in spleen cells after CVB4 infection, treated with Rupintrivir for 24 h. Hoechst 33342 was used for nuclear labeling (blue fluorescence). Rabbit anti-mouse IgM-FITC secondary antibodies were used for p-p38 detection (green fluorescence). (A) 3D-SIM imaging system (DeltaVision OMX) was used for analysis (Bars: 10μm): compared with the viral control group, the distribution of p-p38 in the cytoplasm has no significant difference after treated with Rupintrivir. (B) Reconstruction of p-p38 images from (A) confirmed p-p38 was mainly located in the cytoplasm. (Bars: 2μm). (C) Representative histograms are shown for p-p38 percentage in the nucleus.

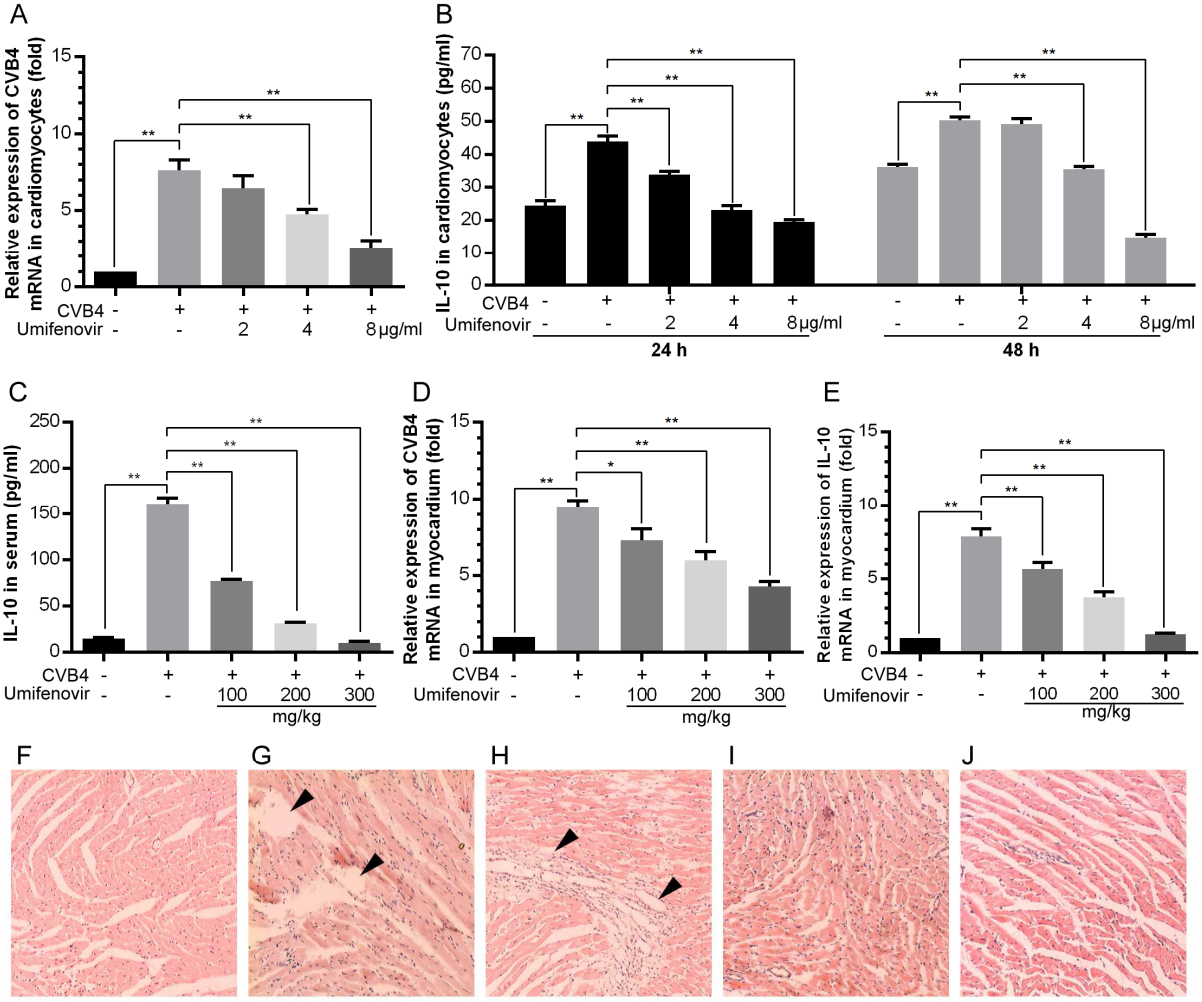
Fig. 6. Analysis of p-MK2 localization in spleen cells after CVB4 infection, treated with Rupintrivir for 24 h. Hoechst 33342 was used for nuclear labeling (blue fluorescence). Alexa Fluor 568 donkey anti-rabbit IgG secondary antibodies were used for p-MK2 detection (red fluorescence). (A) 3D-SIM imaging system (DeltaVision OMX) was used for analysis (Bars: 10μm): compared with the viral control group, the distribution of p-MK2 in the nucleus has no significant difference after treated with Rupintrivir. (B) Reconstruction of p-MK2 images from (A) confirmed p-MK2 was mainly located in the nucleus (Bars: 2μm). (C) Representative histograms are shown for

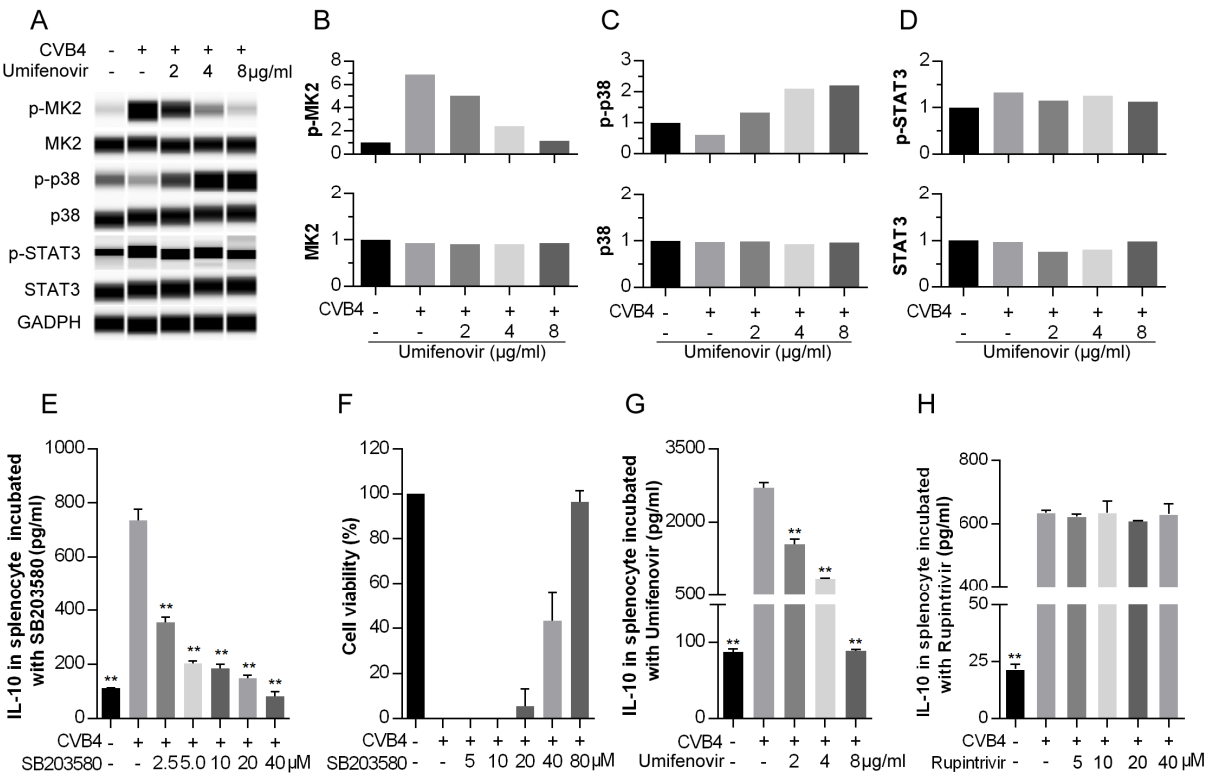
p-MK2 percentage in the nucleus.

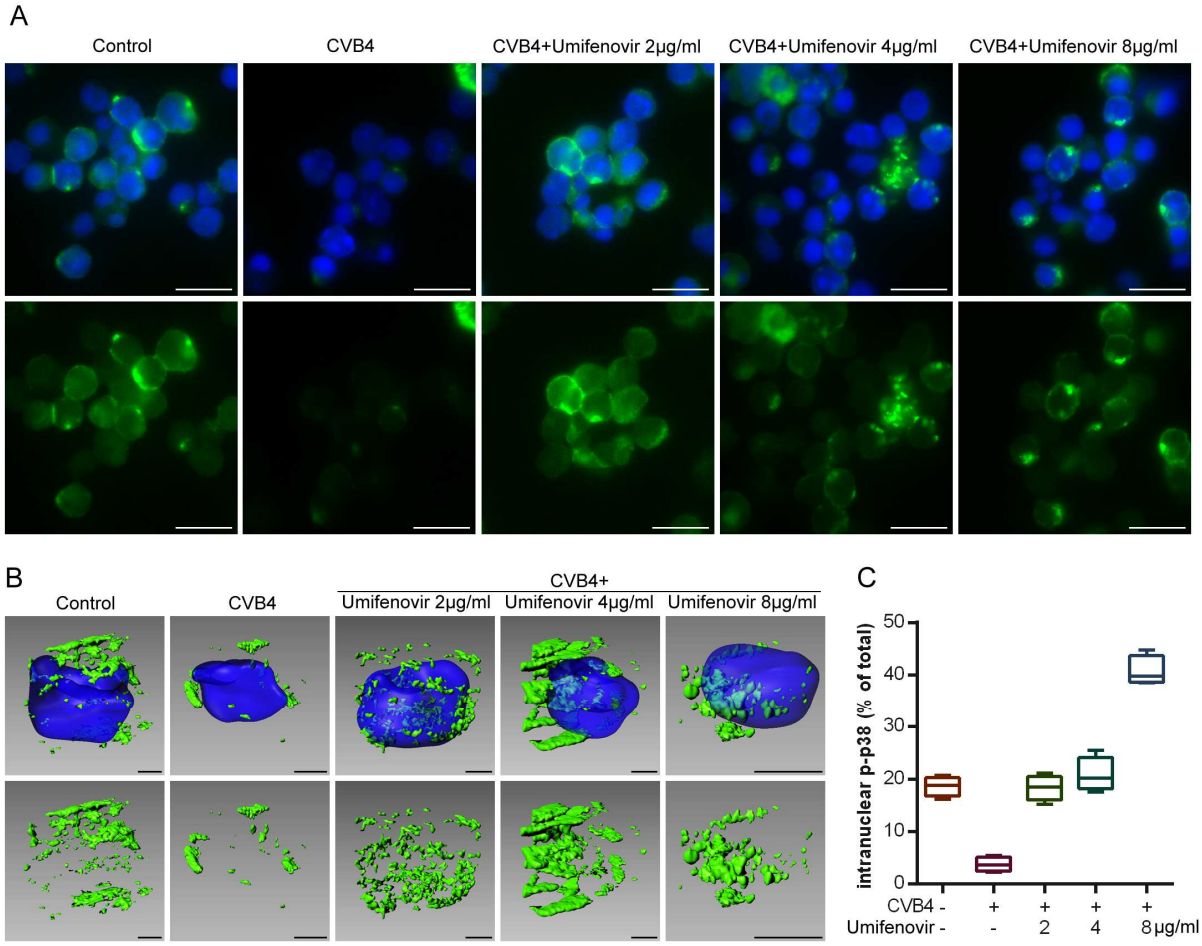
Fig. S1 Myocardial cells collected from heart tissues of newborn BALB/c mice (1-2 days old) were seeded in a 96-well at 1.5×104 cells/well, incubated with Umifenovir(2 μg/ml, 4 μg/ml, 6 μg/ml, 8 μg/ml, 10 μg/ml, 12 μg/ml, 14 μg/ml, 16 μg/ml, 18 μg/ml, 20 μg/ml) for 72 h.The cell viability was measured in WST-1. And the CC50 value was found to be 16.29 μg/ml and the CC0 value was found to be 8 μg/ml.

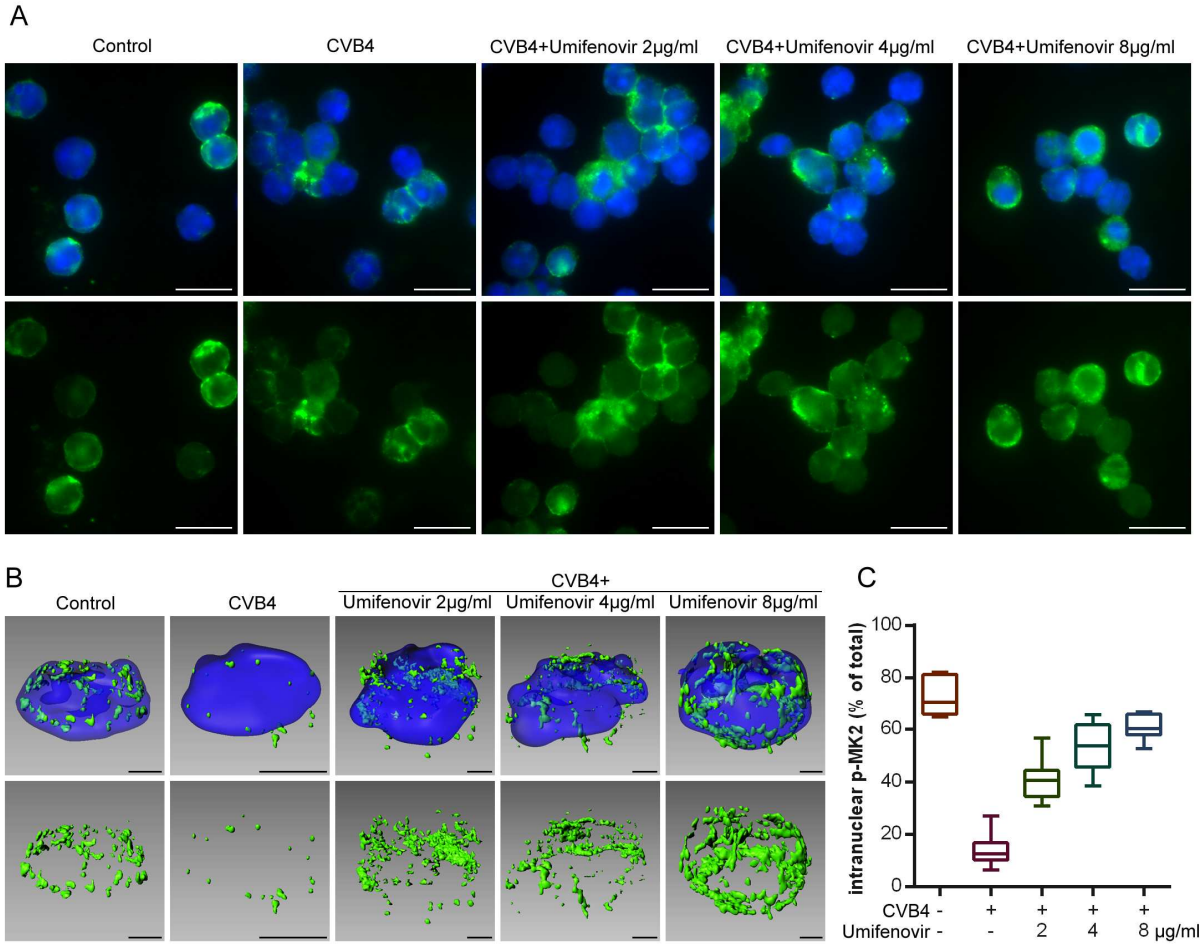
Fig. S2 Spleen cells were obtained from BALB/c mice incubated with LPS (1 μg/ml), adding different doses of Umifenovir (2, 4, and 8 μg/ml). In the normal- and LPS- control groups, the same volume of culture medium was added. Supernatants was collected 24 h later, and the level of IL-10 was measured by ELISA.

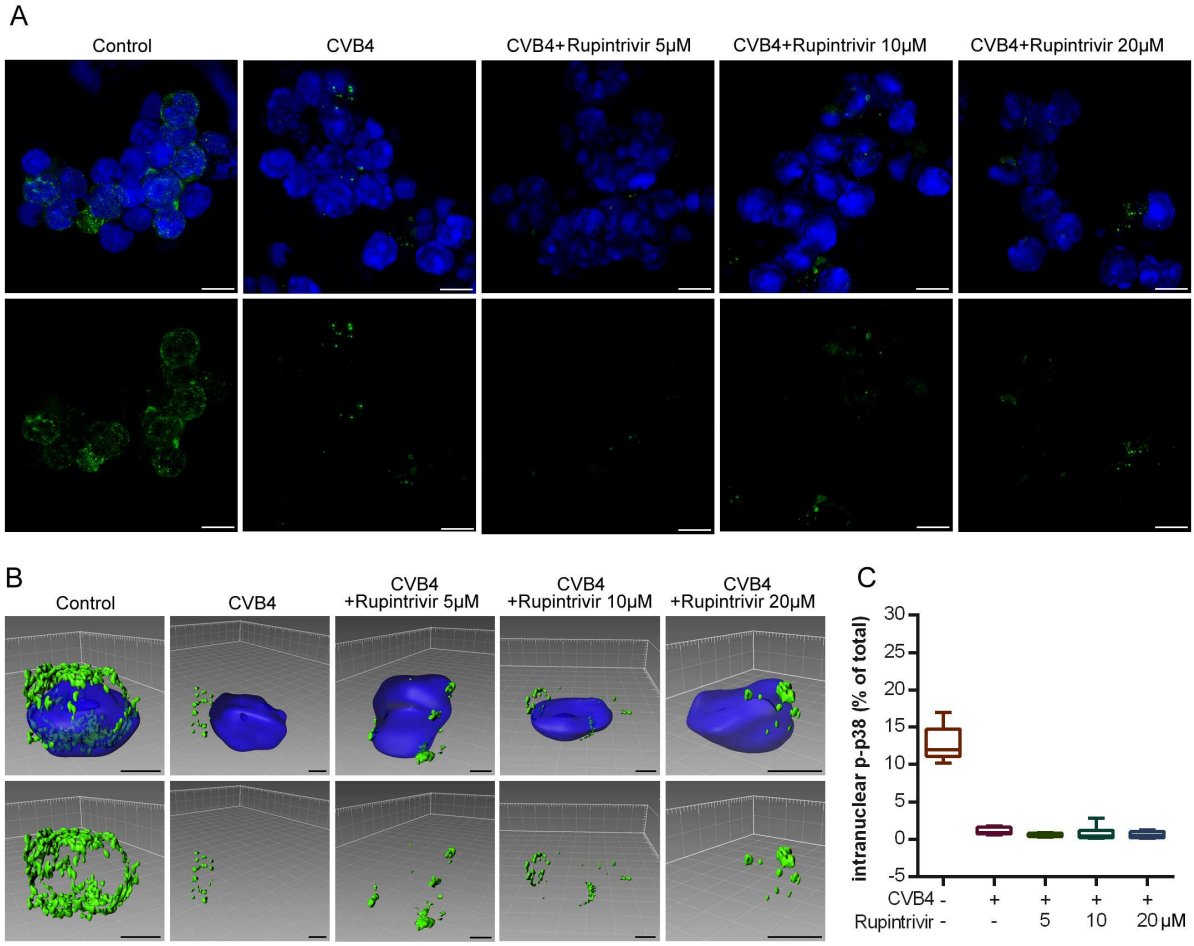
Fig. S3 Hela cells were seeded in 96-well at 1×10^4 cells/well, infected with CVB4 (2.8×10^3TCID50 and treated with Rupintrivir (5 μM, 10 μM, 20 μM, 40 μM, 80 μM) the following day. After 48 h incubation, the cell viability was measured in WST-1.

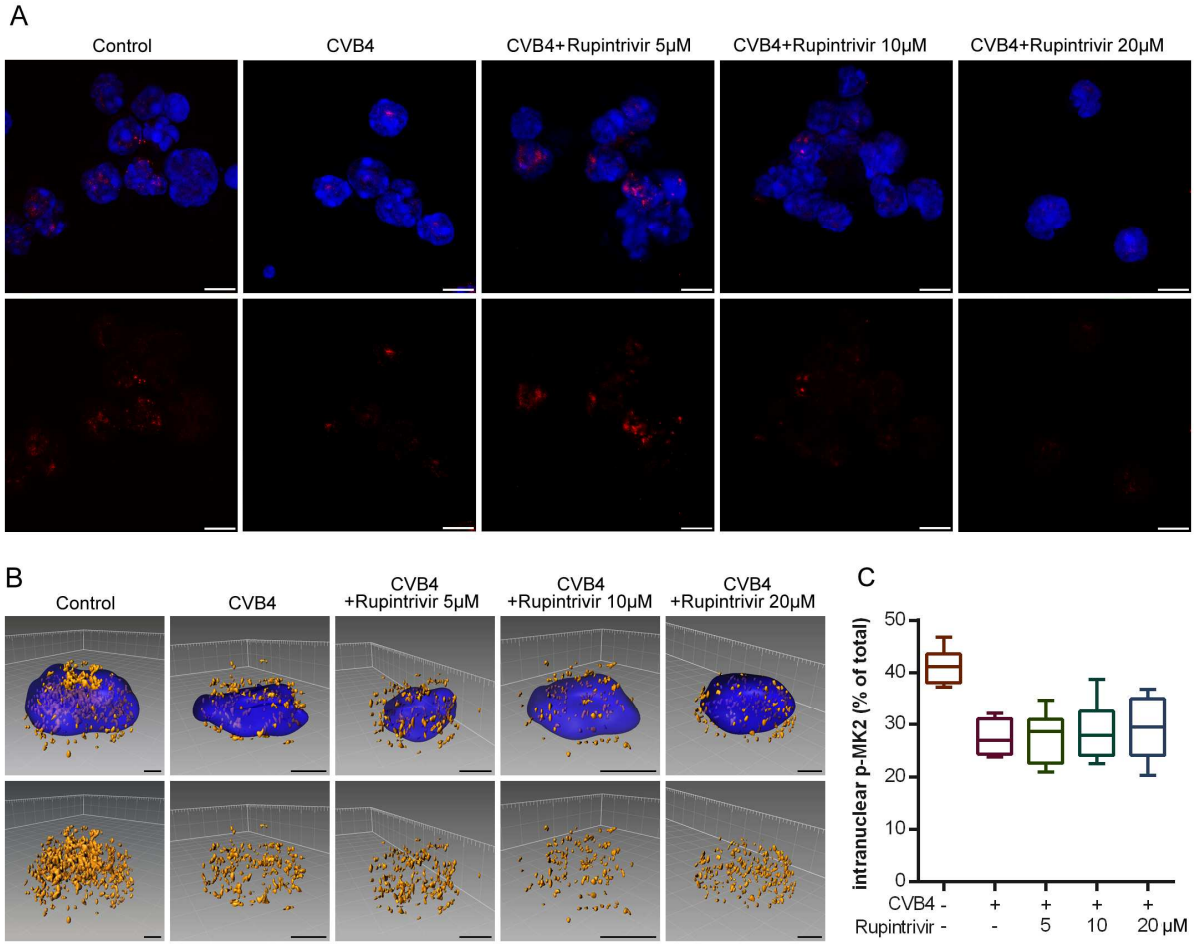

A
Relative expression of CVB4 mRNA in cardiomyocytes (fold)
CVB4
Umifenovir
2
4
8μg/ml
B
IL-10 in cardiomyocytes (pg/ml)
24 h
48 h
C
IL-10 in serum (pg/ml)
mg/kg
D
Relative expression of CVB4 mRNA in myocardium (fold)
E
Relative expression of IL-10 mRNA in myocardium (fold)
F
G
H
I
J




A
Control
CVB4
CVB4+Umifenovir 2μg/ml
CVB4+Umifenovir 4μg/ml
CVB4+Umifenovir 8μg/ml
B
Control
CVB4
CVB4+
Umifenovir 2μg/ml
Umifenovir 4μg/ml
Umifenovir 8μg/ml
C
intranuclear p-p38 (% of total)
50
40
30
20
10
0
CVB4 - + + + +
Umifenovir - - 2 4 8μg/ml


A
Control
CVB4
CVB4+Umifenovir 2µg/ml
CVB4+Umifenovir 4µg/ml
CVB4+Umifenovir 8µg/ml
B
Control
CVB4
CVB4+
Umifenovir 2µg/ml
Umifenovir 4µg/ml
Umifenovir 8µg/ml
C
intranuclear p-MK2 (% of total)
100
80
60
40
20
0
CVB4 - + + + +
Umifenovir - - 2 4 8 µg/ml


A
Control
CVB4
CVB4+Rupintrivir 5μM
CVB4+Rupintrivir 10μM
CVB4+Rupintrivir 20μM
B
Control
CVB4
CVB4 +Rupintrivir 5μM
CVB4 +Rupintrivir 10μM
CVB4 +Rupintrivir 20μM
C
intranuclear p-p38 (% of total)
30
25
20
15
10
5
0
-5
CVB4 - + + + +
Rupintrivir - - 5 10 20 μM


A
Control
CVB4
CVB4+Rupintrivir 5μM
CVB4+Rupintrivir 10μM
CVB4+Rupintrivir 20μM
B
Control
CVB4
CVB4 +Rupintrivir 5μM
CVB4 +Rupintrivir 10μM
CVB4 +Rupintrivir 20μM
C
intranuclear p-MK2 (% of total)
50
40
30
20
10
0
CVB4 - + + + +
Rupintrivir - - 5 10 20 μM

Highlights

1. Dual Role of Umifenovir：antiviral activity and immunomodulating agent.

2. To assess the subcellular localization of p38/p-p38 and MK2/p-MK2 using 3D-SIM imaging.

3. Umifenovir prevented p38-MK2 complex from exiting the cell nucleus.