Roles of stress-activated protein kinases in the replication of Singapore grouper iridovirus and regulation of the inflammatory responses in grouper cells

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Stress-activated protein kinases (SAPKs), including p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK), are usually activated in response to different environmental stimuli, including virus infection. In the present study, the roles of SAPKs during Singapore grouper iridovirus (SGIV) infection were investigated in fish cells. The results showed that increased phosphorylation of JNK1/2 and p38 MAPK occurred during active replication of SGIV in grouper cell cultures. Moreover, downstream effectors (c-Jun, MAPK-activated protein kinase 2, p53, activator protein 1, Myc and nuclear factor of activated T cells) were activated after SGIV infection, suggesting that SGIV replication activated the JNK and p38 MAPK signalling pathways. Notably, using specific inhibitors, it was found that viral gene transcripts, protein expression and viral titres were not affected by inhibition of p38 MAPK but were suppressed significantly by inhibiting JNK1/2 activation. In addition, transcription of grouper immune genes including interferon regulatory factor 1, interleukin-8 and tumour necrosis factor alpha (TNF-α) were regulated by JNK, whilst only TNF-α was regulated by p38 MAPK. It is proposed that the JNK pathway is important for SGIV replication and modulates the inflammatory responses during virus infection.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascades are conserved multifunctional signalling pathways that regulate cell growth, inflammatory responses, apoptosis and cellular responses to different stimuli (Dong et al., 2002). Among the four subgroups of the MAPK family, p38 MAPK and c-Jun N-terminal kinase (JNK), termed stress-activated protein kinases (SAPKs), are usually induced by various stimuli, including viruses, toxins, environmental stresses and inflammatory cytokines (Holloway & Coulson, 2006; Si et al., 2005; Tibbles & Woodgett, 1999). Distinct upstream activators phosphorylate SAPKs and then activate downstream transcription factors, including c-Jun, activating transcription factor 2 (ATF-2) and activator protein 1 (AP-1) (Junttila et al., 2008; Zhang & Liu, 2002; Dong et al., 2002). Ultimately, activation of these transcription factors induces various defence responses by regulation of gene expression.

Viruses can exploit various intracellular signalling pathways to induce cellular and/or viral gene expression for completion of their life cycle. Activation of SAPKs by virus infection plays crucial roles in virus replication and viral progeny release (Wei et al., 2009; Mori et al., 2003; Rezaee et al., 2006; Si et al., 2005; Rahaus et al., 2004). Phosphorylation of SAPKs is increased during replication of coxsackievirus B3-infected cells, but only inhibition of JNK1/2 resulted in a significant reduction in viral progeny release and suppression of virus-induced cell death (Si et al., 2005). In contrast, SAPKs are also activated in encephalomyocarditis virus (EMCV) infection, but only p38 MAPK plays a critical role in the replication of EMCV by regulating the translation of viral RNA (Hirasawa et al., 2003). The JNK signalling pathway is activated during mouse hepatitis virus (MHV) infection, and inhibition of JNK phosphorylation was found to modulate the production of the pro-inflammatory cytokines tumour necrosis
Roles of SAPKs in SGIV replication

RESULTS

Activation of JNK and p38 MAPK during the SGIV replication cycle

In order to investigate whether SGIV infection causes activation of p38 MAPK and JNK, Western blot and immunofluorescence staining studies were performed. Microscopic observation showed that p38 MAPK phosphorylation was observed at 1 h post-infection (p.i.), and obvious nuclear localization increased up to 24 h p.i. compared with mock-infected cells. Notably, intense fluorescent signals were observed mostly at 24 h p.i. when virus assembly sites were formed (Fig. 1a). Consistent with the fluorescence microscopy, the Western blot results showed that p38 MAPK phosphorylation occurred at 1 h p.i. and increased up to 24 h p.i. compared with mock-infected cells (Fig. 1c). In contrast, SGIV infection induced JNK phosphorylation at 2 h p.i. but not at 24 h p.i. or in mock-infected cells (Fig. 1b). The results of Western blotting showed that phosphorylated JNK could be detected from 1 to 6 h p.i. but not at the late stage of infection. The increasing expression of SGIV structural proteins ORF072 and ORF016 with infection time revealed that SGIV replicated well in grouper spleen (EAGS) cells. No changes were observed in the total amounts of JNK, p38 MAPK and β-actin proteins (Fig. 1c). These results demonstrated that both JNK and p38 MAPK were activated during SGIV infection in grouper cells.

Downstream targets of JNK and p38 MAPK are activated during SGIV infection

JNK and p38 MAPK activation contributes to the phosphorylation and activation of the downstream targets, including transcription factors c-Jun, ATF-2 and MAPK-activated protein kinase 2 (MAPKAPK-2), which in turn regulate the transcription of a multitude of cellular genes. In contrast to the time course of JNK activation, c-Jun showed a biphasic activation profile, and levels of phosphorylated c-Jun were decreased at 6 h p.i. (Fig. 2a). Increased phosphorylation of c-Jun was also observed in nuclei at 2 h p.i. (Fig. 2b). Interestingly, the intense fluorescence was congregated and co-localized with virus assembly sites at 18 h p.i., whereas the fluorescence in nuclei was reduced. At 24 h p.i., the fluorescence at virus assembly sites increased, whilst that in the nuclei continued to decrease (Fig. 2b). Consistent with the activation of p38, MAPKAPK-2 phosphorylation occurred from 18 to 48 h p.i. However, ATF-2 phosphorylation was not detected during SGIV infection (Fig. 2a). No activation of c-Jun or MAPKAPK-2 was detected in mock-infected cells.

We also examined the activation of other transcription factors – p53, AP-1, nuclear factor of activated T-cells (NFAT) and c-Myc – using luciferase reporter gene assays. Time-dependent gene expression of p53, AP-1, NFAT and c-Myc increased significantly following SGIV infection. Of note, SGIV infection resulted in approximate increases of 311-fold in c-Myc activation, 544-fold in p53 activation, 42-fold in NFAT activation and fourfold in AP-1 activation at 48 h p.i. (Fig. 3). These results suggested that SGIV infection activated the downstream targets of JNK and p38 MAPK signalling pathway.

Effects of inhibition of SAPKs on SGIV production

To determine the effect of the activation of SAPKs on the replication of SGIV, we examined the impact of these kinases on progeny virus production using SAPK inhibitors. The concentrations of inhibitors used in this study showed no detectable toxicity on EAGS cells (Fig. 4a). Treatment with JNK inhibitor (20 μM SP600125) delayed the cytopathic effects (CPE) induced by SGIV at 48 h p.i., whereas no obvious effects on the presence of CPE were observed after the addition of p38 MAPK inhibitor (20 μM SB203580) (Fig. 4b). Viral titres in culture supernatants or cell lysates were also determined in the presence of SAPKs inhibitors. After treatment with 20 μM SB203580, the viral titre from the cell lysates increased 0.7-fold compared with that from DMSO-treated cells. No obvious changes occurred in viral titre from the supernatants after treatment with 20 μM SB203580. In contrast, after

factor alpha (TNF-α) and interleukin-6 (IL-6) (Yu et al., 2009). The different activation patterns of SAPKs and their roles in replication illustrate the complexity of the regulatory mechanism in virus infections.

Groupers (Epinephelus spp.) are widely cultured in Southeast Asian countries. However, the emergence of viral pathogens, including iridovirus and nervous necrosis virus, has caused heavy economic losses in grouper aquaculture (Qin et al., 2001; Hegde et al., 2002). Singapore grouper iridovirus (SGIV) is a ranavirus belonging to family Iridoviridae and isolated from diseased groupers (Qin et al., 2001, 2003). Our previous studies revealed that SGIV infection in grouper cells evoked a novel type of programmed cell death that is different from the typical apoptosis triggered by other iridovirus isolates (Huang et al., 2006, 2007, 2009; Chinchar et al., 2003). To date, only the JNK signalling pathway has been confirmed to be involved in iridovirus infection using a JNK-specific inhibitor (Chitnis et al., 2008), whilst the role of the MAPK signalling pathway in iridovirus replication remains unknown.

To elucidate the potential roles of SAPKs in the replication cycle of SGIV, we examined the activation of JNK and p38 MAPK during SGIV infection, and the effect of their inhibitors on SGIV replication. We have shown for the first time that the JNK and p38 MAPK signalling pathways are involved in SGIV infection by modulating the inflammatory response. These findings shed new light on the molecular mechanisms of iridovirus pathogenesis.
inhibition of JNK phosphorylation by 20 μM SP600125, the viral titre in supernatants and lysates was decreased by five- and 13-fold compared with those from DMSO-treated cells, respectively (Fig. 4c). These results indicated that the JNK pathway plays a crucial role in the production of SGIV.

**JNK, but not p38 MAPK, is important for SGIV transcription and protein synthesis**

To explore further the role of JNK and p38 MAPK in SGIV replication, we examined the effects of these inhibitors on the transcription and expression of viral genes. Three genes of SGIV, ORF016, ORF019 and ORF072, were studied. As shown in Fig. 5(a), quantitative real-time RT-PCR (qRT-PCR) analysis showed that no significant changes in the transcription levels of SGIV ORF016, ORF019 and ORF072 were detected after the addition of 10 or 20 μM SB203580 compared with DMSO-treated infected cells. In contrast, inhibition of JNK by 10 or 20 μM SP600125 significantly decreased the transcription levels of these three genes. Specifically, treatment with 20 μM SP600125 resulted in a 10-, 16- and 20-fold decrease in the relative abundance of ORF019, ORF016 and ORF072 transcripts, respectively.

Synthesis of viral structural proteins was analysed in the presence and absence of inhibitors by Western blotting. No significant differences were observed in the protein synthesis of SGIV ORF016, ORF019 and ORF072 between DMSO-treated infected cells and 20 μM SB203580-treated infected cells. In contrast, the band density of these three proteins was clearly reduced after treatment with 20 μM SP600125 (Fig. 5b). These results suggested that inhibition...
of the JNK pathway decreases viral gene transcription and protein synthesis during SGIV infection, whereas p38 MAPK inhibition has no marked effect.

**Effect of SAPKs inhibitors on regulation of fish immune-response genes**

To investigate whether fish interferon regulation factor 1 (IRF-1), IL-8 and TNF-α production was regulated by SGIV-induced SAPK activation, EAGS cells were treated with 20 μM SB203580 or SP600125 (or DMSO as a control) for 1 h before inoculating with SGIV, and transcription of IRF-1, IL-8 and TNF-α was examined by qRT-PCR. SGIV infection evoked increased transcription of IRF-1, IL-8 and TNF-α in EAGS cells (Fig. 6). Inhibition of JNK by 20 μM SP600125 resulted in a significant reduction in IL-8, TNF-α and IRF-1 production, whereas inhibition of p38 MAPK by 20 μM SB203580 decreased only TNF-α production. These results indicated that fish immune genes are regulated by SAPKs during virus infection.

**DISCUSSION**

Virus infections are known to activate various cellular signalling pathways, which could affect cellular function and facilitate virus replication. In mammals, both p38 MAPK and JNK are activated in response to a variety of pro-inflammatory cytokines and various environmental stimuli, including virus infection (Rahaus *et al.*, 2004; Wei *et al.*, 2009). In this study, we observed p38 MAPK phosphorylation in EAGS cells throughout SGIV infection, and activation of p38 MAPK increased during virus replication. Downstream effectors, including MAPKAPK-2 and transcription factors Myc, p53, NFAT and AP-1, were also activated, suggesting that the p38 MAPK signalling pathway participated in SGIV infection. Activation of the p38 MAPK signalling pathway has also been confirmed in Kaposi’s sarcoma-associated herpesvirus and human cytomegalovirus infections (Pan *et al.*, 2006; Johnson *et al.*, 2000). Phosphorylation of JNK1/2 was also detected in the early stage of SGIV infection. Intriguingly, c-Jun phosphorylation in nuclei increased significantly during the early stage of SGIV infection and decreased at the late stage, but a phosphorylation signal also appeared and increased, accompanied by the appearance and enlargement of virus assembly sites. Transcription factor c-Jun is subject to multiple forms of regulation, including modulating cell proliferation and cell death, in response to different extracellular stimuli (Bossy-Wetzel *et al.*, 1997). Whether c-Jun fulfils a novel function by interacting with virus structural proteins during SGIV infection should be investigated in further studies.

Although SAPKs can be activated in response to different viruses, the detailed roles of the SAPK pathways in virus replication are intricate. Inhibition of JNK1/2 and p38 kinases resulted in a significant reduction in porcine circovirus type 2 viral protein synthesis, viral progeny release and virus-induced apoptosis (Wei *et al.*, 2009). In the case of DNA viruses, inhibition of JNK resulted in a
twofold increase in varicella-zoster virus replication, whereas a strong decrease in virus replication was observed after inhibition of p38 MAPK (Rahaus et al., 2004). Treatment of EAGS cells with the JNK inhibitor SP600125 reduced virus production significantly and delayed the appearance of CPE. The reduction in virus production might be directly due to a decrease in viral protein synthesis and viral gene transcription, suggesting that JNK signalling contributes greatly to the replication cycle of SGIV. Given that the JNK pathway activates AP-1 components to regulate cytoskeleton organization (Martin-Blanco et al., 2000), and that cytoskeleton disruption and rearrangement have also been reported in iridovirus infections (Huang et al., 2009; Murti & Goorha, 1983), we speculate that the JNK pathway could regulate SGIV infection by modulating cytoskeleton action. Although p38 MAPK was activated at the late stage of SGIV infection, virus replication was not impaired by the p38 MAPK inhibitor SB203580. The exact role of p38 MAPK during SGIV infection needs further investigation.

The phosphorylation and activation status of kinases in the MAPK system has crucial impacts on infection-induced expression of immune-response genes (Liu et al., 2007; Saloijn & Oravecz, 2007). Activation of the p38 MAPK pathway was found to be responsible for the production of the pro-inflammatory cytokines TNF-α and IL-1β, which are key contributors to the pathological process in cats, after infection with feline infectious peritonitis virus (Regan et al., 2009). Alternatively, the induction of TNF-α and IL-6 by MHV strain A59 infection is mediated through activation of the JNK signalling pathway but not through the extracellular signal-regulated kinase or p38 signalling pathway (Yu et al., 2009). Our results indicated that inhibition of JNK activation significantly reduced the transcription of TNF-α, IL-8 and IRF-1, whilst inhibition of p38 MAPK activation resulted in a specific decrease in TNF-α level. It has been demonstrated that TNF-α, IL-8 and IRF-1 play important roles in cell fate determination (Varfolomeev & Ashkenazi, 2004; Abdollahi et al., 2005; Chapman et al., 2000), and that programmed cell death is evoked during SGIV infection in multiple grouper cells (Huang et al., 2009, 2011). These gene expression changes mediated by SAPKs could contribute to the cell death resulting from SGIV infection. Moreover, IRFs and pro-inflammatory cytokines play crucial roles in inflammation.

![Fig. 3. AP-1-, p53-, NFAT- and c-Myc-dependent luciferase expression in SGIV-infected EAGS cells. Cells were co-transfected with 0.4 μg pCMV-β-gal and 1 μg of the luciferase reporter constructs pMyc-Luc (a), p53-Luc (b), pNFAT-Luc (c) or pAP1-Luc (d) and then infected with SGIV and incubated for the indicated time intervals. Cell extracts were prepared and luciferase (LUC) activity was measured. β-Galactosidase activity was detected to normalize for transfection efficiency. Experiments were carried out independently three times. Results are shown as means ± sd. *, P<0.05; **, P<0.01.](image-url)
and the antiviral immune response, and are regulated by SAPKs in response to different viruses (Tamura et al., 2008; Li et al., 2006; Xie et al., 2005; Cheung et al., 2002; Taniguchi et al., 2001). In addition to the pro-inflammatory cytokines, SGIV-activated SAPK pathways might modulate the expression and modification of many other cellular proteins to facilitate virus infection.

In summary, our findings revealed for the first time that activation of the JNK and p38 MAPK pathways occurs during fish virus infection. The requirement for JNK in SGIV replication and its regulatory roles on immune-related genes have unveiled the complexity of the regulatory mechanism between virus replication and intracellular signalling transduction.

**METHODS**

**Virus and cell.** EAGS cells were grown and maintained in Leibovitz’s L-15 medium containing 10% FBS (Gibco) at 25 °C (Huang et al., 2009). SGIV (strain A3/12/98 PPD) was propagated in EAGS cells and stored at −80 °C (Qin et al., 2001). Cells were infected with SGIV at an m.o.i. of 2.

**Regents and antibodies.** SP600125 and SB203580 (Alexis Corporation), inhibitors of JNK and p38 MAPK, respectively, were dissolved in DMSO. EAGS cells were treated with either 0.1% DMSO or various concentrations (10 and 20 μM) of these two inhibitors for 1 h prior to infection. The cytotoxicity of the inhibitors on EAGS cells was determined by trypan blue exclusion dye staining. Primary antibodies anti-phospho-p38 MAPK, anti-phospho-SAPK/JNK, anti-phospho-MAPKAPK-2, anti-phospho-ATF-2, anti-phospho-c-Jun and anti-SAPK/JNK were purchased from Cell Signaling Technology. Anti-β-actin mAb was purchased from Abcam, and the polyclonal rabbit antibody against p38 was from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies and rhodamine-conjugated goat anti-rabbit IgG were purchased from Pierce Chemical Corp.

**Western blot analysis.** At the indicated time points p.i., mock- and SGIV-infected cells were harvested and the pellets were resuspended in PBS. Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 h with 5% non-fat dried milk solution and incubated with the primary antibodies overnight at 4 °C. Except for antibodies against viral protein, the primary antibodies were diluted 1:1000 in Tris-buffered saline containing 5% BSA and 0.1% Tween 20 (TBST). After washing with TBST, the membranes were incubated for an additional hour with the secondary antibody, which was HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG at a dilution of 1:1000. Simultaneously, internal controls were performed by detecting β-actin. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham ECL Western Blotting Detection kit; GE Healthcare) and the immunoblots for virus proteins were visualized with diaminobenzidine.

**Immunofluorescence.** EAGS cells were grown on coverslips in six-well plates. Mock- and SGIV-infected cells were fixed in 4% paraformaldehyde and permeabilized with 100% alcohol for 7 min at −20 °C. After washing with PBS, cells were blocked with 2% BSA for 30 min and then incubated with primary antibodies (1:100) for 2 h at room temperature. The coverslips were washed with PBS and incubated with the secondary antibody, rhodamine-conjugated goat anti-rabbit IgG. Nuclei were stained with DAPI (1 μg ml⁻¹; Sigma) for 10 min. Samples were examined under an inverted fluorescence microscope (Leica).

**Reporter gene assay.** EAGS cells in 24-well tissue-culture plates were cultured for 18 h before being transfected with 1 μg luciferase reporter gene plasmid. EAGS cells were grown and maintained in Leibovitz’s L-15 medium containing 10% FBS (Gibco) at 25 °C (Huang et al., 2009). SGIV (strain A3/12/98 PPD) was propagated in EAGS cells and stored at −80 °C (Qin et al., 2001). Cells were infected with SGIV at an m.o.i. of 2.

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reporter construct (p53-Luc, pAP-1-Luc, pNFAT-Luc or pMyc-Luc) and 0.4 µg pCMV-β-gal reporter construct (Clontech) using Lipofectamine 2000 (Invitrogen). After treatment with DMSO, SB203580 or SP600125 for 1 h, EAGS cells were incubated with SGIV at an m.o.i. of 2 for 48 h. Cells were collected for further RNA extraction and qPCR-PAR examination. The relative transcription of viral genes in DMSO-treated cells following SGIV infection was defined as 1, and that in SB203580- or SP600125-treated EAGS cells with SGIV infection was indicated by fold increase or decrease compared with the control. Experiments were carried out independently three times. Results are presented as means ± SD. *, P<0.05; **, P<0.01. (b) Effect of 20 µM SB203580 or SP600125 on synthesis of SGIV structural proteins ORF016, ORF019 and ORF072. After treatment with DMSO, SB203580 or SP600125 for 1 h, EAGS cells were incubated with SGIV at an m.o.i. of 2 at the indicated time points (24 and 48 h p.i.). Cells were collected and Western blotting was performed using antibodies against β-actin and SGIV ORF016, ORF019 and ORF072. Experiments were carried out independently three times. The results presented are from one representative experiment.

**Fig. 5.** Effect of SAPK inhibitors on viral gene transcription and viral protein synthesis. (a) qRT-PCR detection of viral gene transcription in SGIV-infected EAGS cells after treatment with 10 or 20 µM SB203580 or SP600125. After treatment with DMSO, SB203580 or SP600125 for 1 h, EAGS cells were incubated with SGIV at an m.o.i. of 2 for 48 h. Cells were collected for further RNA extraction and qPCR examination. The relative transcription of viral genes in DMSO-treated cells following SGIV infection was defined as 1, and that in SB203580- or SP600125-treated EAGS cells with SGIV infection was indicated by fold increase or decrease compared with the control. Experiments were carried out independently three times. Results are presented as means ± SD. *, P<0.05; **, P<0.01. (b) Effect of 20 µM SB203580 or SP600125 on synthesis of SGIV structural proteins ORF016, ORF019 and ORF072. After treatment with DMSO, SB203580 or SP600125 for 1 h, EAGS cells were incubated with SGIV at an m.o.i. of 2 at the indicated time points (24 and 48 h p.i.). Cells were collected and Western blotting was performed using antibodies against β-actin and SGIV ORF016, ORF019 and ORF072. Experiments were carried out independently three times. The results presented are from one representative experiment.

**Determination of viral titres in supernatants or cell lysates.**
Viral titres in culture supernatants or cell lysates were determined on monolayers of EAGS cells by an agar overlay plaque assay in triplicate. Supernatants were removed from cultures at 48 h p.i. and frozen at −80 °C until assayed for viral titre. Adherent cells were lysed in 500 µl fresh medium by freezing and thawing three times (lysate). Samples were serially diluted tenfold, overlaid on 95% confluent monolayers of EAGS cells in 24-well plates and incubated for 1 h. Medium containing virus was removed, and cells were washed with fresh medium three times. Complete L-15 medium was added to the wells after 15 min and the cells were incubated at 25 °C for 5 days. Plaque number was counted by microscopy and the viral titre was calculated as p.f.u. ml⁻¹.
Extraction of RNA and reverse transcription. Total RNA was extracted using an SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions. DNA contamination was removed by incubation in RNase-free DNase I for 15 min, and the RNA was dissolved in nuclease-free water. Reverse transcription reactions were performed on 2 μg total RNA with a ReverTra Ace qPCR RT kit (Toyobo). Briefly, mixtures containing 2 μg total RNA, 1 μl reverse transcriptase mix, 1 μl primer mix and 4 μl 5X reverse transcriptase buffer in a total volume of 20 μl were incubated at 37 °C for 15 min, 98 °C for 5 min and 4 °C for 5 min. The resulting cDNA products were aliquotted and stored frozen at −70 °C until use.

qRT-PCR analysis. To detect whether the transcription levels of viral genes were altered after treatment with inhibitors of p38 MAPK and JNK, qRT-PCR was used to evaluate the relative RNA expression of SGIV genes encoding structural proteins, including the major capsid protein (ORF072) and envelope proteins (ORF016 and ORF019). Briefly, SGIV-infected EAGS cells treated with 0.1 % DMSO or 10 or 20 μM SP600125 or SB203580 were inoculated with SGIV for 48 h and total RNA was extracted as described above. SYBR Green real-time PCR mix (Toyobo) was used for PCR, with a primer concentration of 100 nM. PCR amplification was performed in triplicate wells, using the following cycling parameters: 95 °C for 3 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C and 15 s at 68 °C.
The results were expressed as fold increase in RNA levels compared with SGIV-infected DMSO-treated cells after normalization to β-actin levels. To reveal whether immune-related genes were modulated by SAPKs during SGIV infection, transcripts of IL-8, TNF-α and IRF-1 were also detected in the presence or absence of inhibitors. In brief, DMSO or SAPK inhibitor-treated EAGS cells were inoculated with SGIV at an m.o.i. of 2. At the indicated time points (6 and 24 h p.i.), mock-or SGIV-infected cells were collected for RNA extraction. The relative expressions of IL-8, TNF-α and IRF-1 were detected using qRT-PCR. The results were expressed as fold increase in RNA levels compared with treated uninfected cells after normalization to β-actin.

The primers used in this study are listed in Supplementary Table S1 (available in JGV Online). Data are expressed as means ± S.D. Statistical analysis was performed with one-way analysis of variance and then subjected to Student’s t-test. Differences were considered statistically significant at P<0.05. All the data in this study are representative of three independent experiments.

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