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RESEARCH ARTICLE

The Scorpion Venom Peptide Smp76 Inhibits Viral Infection by Regulating Type-I Interferon Response

Zhenglin Ji[1] · Fangfang Li[1] · Zhiqiang Xia[1] · Xingchen Guo[1] · Minjun Gao[1] · Fang Sun[1] · Yuting Cheng[1] · Yingliang Wu[1,2] · Wenxin Li[1,2] · Syed Abid Ali[3] · Zhijian Cao[1,2,4]

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Abstract

Dengue virus (DENV) and Zika virus (ZIKV) have spread throughout many countries in the developing world and infect millions of people every year, causing severe harm to human health and the economy. Unfortunately, there are few effective vaccines and therapies available against these viruses. Therefore, the discovery of new antiviral agents is critical. Herein, a scorpion venom peptide (Smp76) characterized from *Scorpio maurus palmatus* was successfully expressed and purified in *Escherichia coli* BL21(DE3). The recombinant Smp76 (rSmp76) was found to effectively inhibit DENV and ZIKV infections in a dose-dependent manner in both cultured cell lines and primary mouse macrophages. Interestingly, rSmp76 did not inactivate the viral particles directly but suppressed the established viral infection, similar to the effect of interferon (IFN)-β. Mechanistically, rSmp76 was revealed to upregulate the expression of IFN-β by activating interferon regulatory transcription factor 3 (IRF3) phosphorylation, enhancing the type-I IFN response and inhibiting viral infection. This mechanism is significantly different from traditional virucidal antimicrobial peptides (AMPs). Overall, the scorpion venom peptide Smp76 is a potential new antiviral agent with a unique mechanism involving type-I IFN responses, demonstrating that natural AMPs can enhance immunity by functioning as immunomodulators.

Keywords Dengue virus (DENV) · Zika virus (ZIKV) · Scorpion venom peptide · Smp76 · Antiviral mechanism · Type-I interferon response

Introduction

Dengue virus (DENV) and Zika virus (ZIKV), belonging to the *Flaviviridae* family, are a global threat to human health and result in huge economic losses. DENV and ZIKV are enveloped, positive single-stranded RNA viruses (Chan and Choi 2016) that are mosquito-borne. Due to urbanization and global warming (McMichael *et al.* 2006), the two viruses have spread more widely in tropical and subtropical areas. The incidence of dengue has increased 30-fold over the last 50 years, with 396 million individuals infected every year, an 18% hospitalization rate, and approximately 13,600 deaths (Halstead 2007; Shepard *et al.* 2016). DENV has four serotypes (DENV1–4) that can all result in life-threatening dengue hemorrhagic fever and dengue shock syndrome (Cui *et al.* 2018; Heymann *et al.* 2016; Ubol *et al.* 2008). According to the World Health Organization (WHO), ZIKV is endemic to over 84 countries, territories, or sub-national areas including Africa, the Asia–Pacific regions, and the Americas—particularly

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✉ Zhijian Cao
zjcao@whu.edu.cn

1 State Key Laboratory of Virology, Modern Virology Research Center, College of Life Sciences, Wuhan University, Wuhan 430072, China

2 Bio-drug Research Center, Wuhan University, Wuhan 430072, China

3 International Centre for Chemical and Biological Sciences (ICCBS), HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan

4 Hubei Province Engineering and Technology Research, Center for Fluorinated Pharmaceuticals, Wuhan University, Wuhan 430072, China

South America (Bogoch *et al.* 2016) (http://www.who.int/emergencies/zika-virus-tmp/en/). Infection with ZIKV during pregnancy can lead to congenital Zika syndrome (Alesha Grant *et al.* 2016; Cui *et al.* 2018; Heymann *et al.* 2016), which is a serious health burden to society. Currently, a ZIKV vaccine is not available. Moreover, the tetravalent dengue vaccine (*i.e.*, Dengvaxia) exhibits limited efficacy and can cause severe dengue in naive individuals, thus, it is only licensed in 9–11 countries. There are also no specific therapeutics against DENV and ZIKV (Screaton *et al.* 2015; Yu *et al.* 2017b). Therefore, the discovery of new antiviral agents is urgently needed.

Scorpion venom is a rich source of peptides with a variety of pharmacological functions (Carballar-Lejarazu *et al.* 2008). Antimicrobial peptides (AMPs) are important components of scorpion venom, and some scorpion venom AMPs have been shown to have activity against multiple viral pathogens through different mechanisms (Hong *et al.* 2013; Zeng *et al.* 2018; Zhao *et al.* 2012). For example, Ctry2459 and its derived peptides can inactivate hepatitis C virus (HCV) particles directly (Hong *et al.* 2013), while Eval418 and its derived peptides can suppress herpes simplex virus (HSV)-1 infection by inactivating HSV-1 and inhibiting its attachment to host cells (Zeng *et al.* 2018). Mucroporin-M1 can inhibit hepatitis B virus (HBV) replication by activating the MAPK pathway and down-regulating hepatocyte nuclear factor (HNF4)-α *in vitro* and *in vitro* (Zhao *et al.* 2012). Meanwhile, scorpine was shown to possess anti-DENV activity *in vitro* (Carballar-Lejarazu *et al.* 2008).

Innate immune recognition of viral infection triggers antiviral immune responses. DENV and ZIKV are recognized by pattern recognition receptors (PRRs), particularly retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), that are expressed on innate immune cells (Loo and Gale 2011; Morrison *et al.* 2013). Interferon (IFN)-mediated antiviral responses are important against viral infection and pathogenesis (Schoggins *et al.* 2011; Seth *et al.* 2006; Muller *et al.* 1994). IFN-α/β receptors receive stimulation from type-I IFN, after which hundreds of cellular genes are upregulated, leading to antiviral responses (Jones *et al.* 2005; Morrison *et al.* 2013). Although AMPs are effectors of the innate immune response and can kill bacteria, enveloped viruses, fungi, and tumor cells (Reddy *et al.* 2004), natural AMPs are rarely explored for their regulation of the IFN system, which includes type-I and II IFN immune responses (Biragyn *et al.* 2002; Lande *et al.* 2007).

Smp76, from the venom of the scorpion *Scorpio maurus palmatus*, possesses 76 amino acid residues and is approximately 8397 Da in size (Abdel-Rahman *et al.* 2013). Its N-terminal amino acid sequence is similar to cecropins, whereas its C-terminal region has three pairs of disulfide bridges, sharing the same CSαβ motif with defensins (Renaud Conde *et al.* 2000). Sequence similarity showed that Smp76 belongs to the family of β-KTx-like scorpine from *Pandinus imperator* and HS-1 from *Heterometrus laoticus* (Renaud Conde *et al.* 2000; Uawonggul *et al.* 2014). Considering the high sequence similarity of Smp76 with scorpine and HS-1 (Fig. 1A), we examined the antiviral activity of Smp76, a venom peptide previously characterized from the scorpion *Scorpio maurus palmatus* (Abdel-Rahman *et al.* 2013). In the present study, we implemented a fusion expression and purification strategy in *Escherichia coli* BL21(DE3) to obtain the recombinant venom peptide (rSmp76) and then tested it on cultured cell lines and mouse macrophages to examine its antiviral effects and mechanisms.

Materials and Methods

Cells and Viruses

The human lung adenocarcinoma cell line A549, human hepatoma cell line Huh7, and African green monkey kidney cell line Vero were cultured in Dulbecco's modified Eagle's medium (Gibco DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Gibco FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. The human monocytic cell line THP-1 was cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. THP-1 cells were differentiated to macrophages with 60 nmol/L phorbol-12-myristate-13-acetate (TPA) for 12–14 h and then cultured for 24 h without TPA. The *Aedes albopictus* mosquito cell line C6/36 was cultured at 28 °C without CO_2 in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Peritoneal macrophages were harvested from mice by washing with PFE (PBS supplemented with 5% FBS and 2 mmol/L EDTA) 3 days after injecting the mice intraperitoneally with 4% thioglycollate medium.

DENV serotype-2 *TSV01* strain (DENV-2 *TSV01*) was kindly provided by Dr. Bo Zhang from the Wuhan Institute of Virology, Chinese Academy of Sciences. DENV serotype-2 New Guinea C strain (DENV-2 *NGC*) was kindly gifted by Prof. Jianguo Wu from Wuhan University. The HSV-1 F strain was stored in our laboratory. ZIKV *Puerto Rico* strain (PRVABC59) cDNA plasmid (pBR322-Z2) was kindly provided by Dr. Ren Sun and Dr. Danyang Gong from the University of California, Los Angeles. ZIKV mRNA was obtained by transcription of recombinant plasmid *in vitro*, after which ZIKV mRNA was transfected into Vero cells. Zika virus was harvested 6 days later.

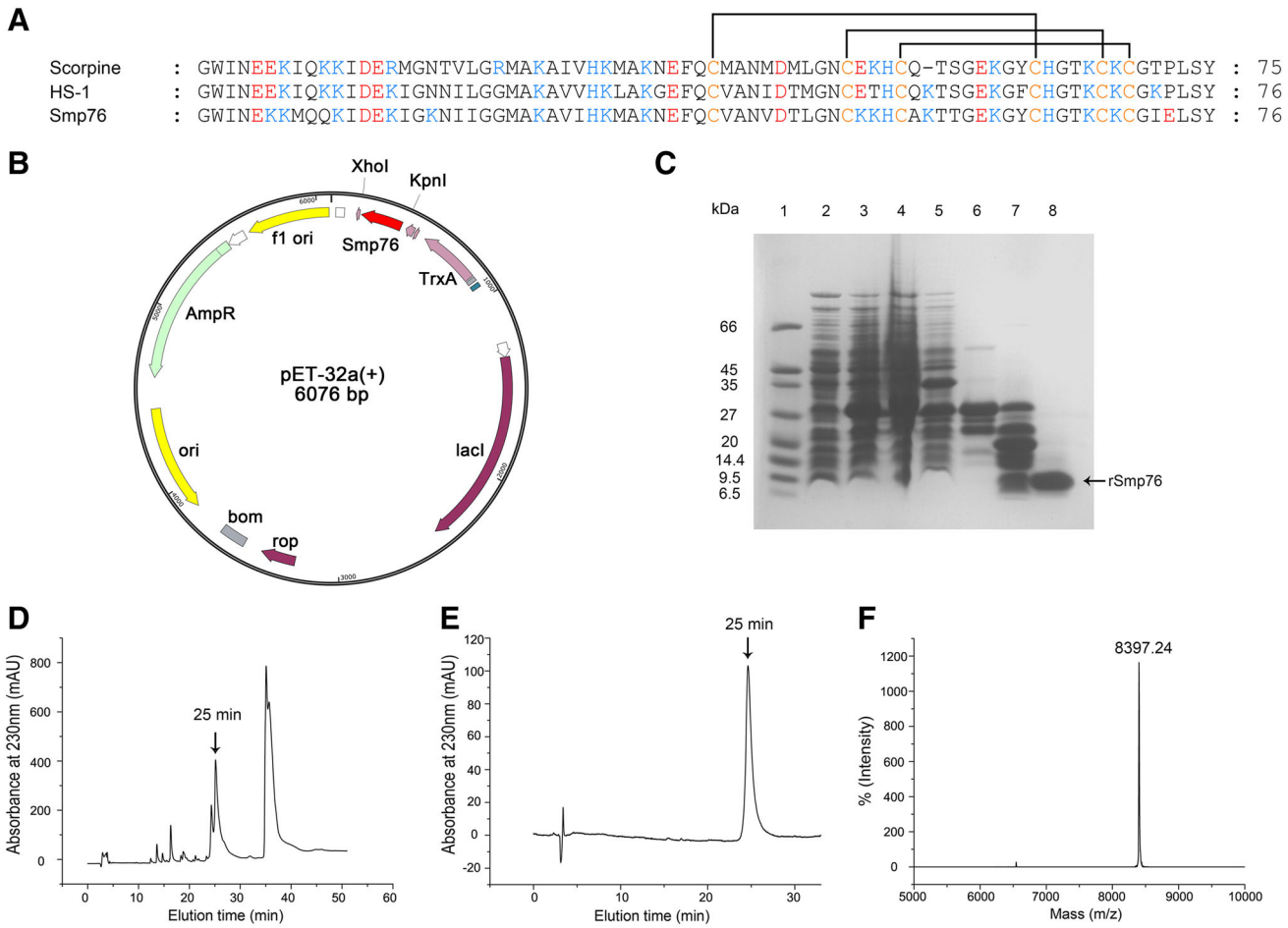

Fig. 1 Expression, purification, and identification of the recombinant peptide Smp76 (rSmp76). **A** Sequence alignment of the three scorpine-like peptides. Highly conserved cysteine residues are marked in yellow font, forming three pairs of disulfide bonds. **B** The cDNA coding sequence of Smp76 mature peptide was cloned into the expression vector pET-32a at the *Kpn* I-*Xho* I restriction sites for cloning and expression of rSmp76 in *E. coli* BL21(DE3). **C** SDS-PAGE of rSmp76 purified from *E. coli* BL21(DE3). Lane 1, protein marker; lane 2, *E. coli* containing pET-32a-rSmp76 without induction; lane 3, *E. coli* induced with IPTG; lane 4, ultrasonicated supernatant from induced *E. coli*; lane 5, ultrasonicated sediment of induced *E. coli*; lane 6, purified His-tag fusion protein after affinity chromatography; lane 7, fusion protein cleaved by enterokinase; lane 8, rSmp76 after HPLC purification. **D** HPLC profile of rSmp76 after cleavage by enterokinase. **E** HPLC purity analysis of rSmp76 with an analytical column. **F** MALDI-TOF-MS mass spectrum of the purified rSmp76.

Mice

Ifnar1$^{-/-}$ mice with a C57BL6/J genetic background were kindly provided by Professor Bo Zhong from Wuhan University. Wild-type C57BL6/J mice were purchased from the Hubei Provincial Center for Disease Control and Prevention, China. Mice were maintained in the specific pathogen-free facility of Wuhan University.

Antibodies and Reagents

Antibodies used were as follows, DENV type 1–4 mouse monoclonal antibody (sc-57,800; Santa Cruz Biotechnology, Dallas, TX), IRF3 rabbit monoclonal antibody (D83B9; Cell Signaling Technologies, Danvers, MA), p-IRF3 rabbit monoclonal antibody (S396; Cell Signaling Technologies), anti-GAPDH mouse antibody (60004-1-Ig; Proteintech, Rosemont, IL), and goat HRP conjugated anti-mouse IgG (GM01H; NovoGene, Beijing, China). Bestar® SybrGreen qPCR master mix reagent was purchased from (DBI® Bioscience, Ludwigshafen, German). Loading sample buffer (5 ×; BL502A) was obtained from Biosharp, Hefei, China. Protein markers used were PageRuler Pre-stained Protein Ladder (26617; Thermo Fisher Scientific) and Protein Marker Low Range (66–4.1 kDa; Kerun Biology, Chongqing, China). The protease and phosphatase inhibitor cocktails MCE HY-K0010 and MCE HY-K0022, respectively, were purchased from MedChemExpress (Monmouth Junction, NJ).

Peptide Expression, Purification, and Characterization

The amino acid sequence of Smp76 peptide was obtained from published data (Abdel-Rahman *et al.* 2013). Smp76 cDNA was reverse translated from the protein sequence. We used overlapping PCR to insert the DNA fragment into the expression plasmid pET-32a with *Kpn* I and *Xho* I restriction enzymes and an enterokinase cleavage site (DDDDK; Fig. 1B). The recombinant vector was then transformed into the chemically competent *Escherichia coli* BL21(DE3). Next, recombinant *E. coli* BL21(DE3) was cultured in LB medium with 100 μg/mL ampicillin at 37 °C and when cell density reached OD_{600} = 0.5, isopropyl β-D-thiogalactoside (IPTG) was added at a final concentration of 1 mmol/L. The cells were then lysed by ultrasonication at 400 Hz for 99 cycles and the supernatant containing the fusion peptide was collected. The obtained supernatant was subjected to Ni-chelating affinity chromatography and the fusion peptide was dialyzed against 1enterokinase buffer for 5 h. Next, the fusion peptide was cleaved with an enterokinase (Morebio, Wuhan, China) overnight at 23 °C and separated by reverse-phased high-performance liquid chromatography (RP-HPLC) using a C18 column (10 × 250 mm, 5 μm; Elite HPLC, Dalian, China). Finally, the target peptide was collected by detecting at a wavelength of 230 nm and immediately freeze-dried. The purity and molecular mass of rSmp76 were confirmed by RP-HPLC and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF–MS), respectively.

RNA Extraction and Quantitative Reverse Transcription (qRT)-PCR

Total RNA of cells or tissues was extracted by TRIzol reagent (TaKaRa, Kusatsu, Japan) and then reverse transcribed to synthesize first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA was quantified by qRT-PCR using the Bestar® SybrGreen qPCR master mix reagent on an ABI 7500 Fast Instrument (Applied Biosystems, Foster City, CA) using standard cycling conditions. The detailed primers for qRT-PCR were in Supplementary Table S1.

Western Blot Analysis

Cells were lysed with RIPA lysis buffer plus the protease and phosphatase inhibitor cocktails on ice. The lysate was centrifuged for 15 min at 14,400 ×*g* and the supernatant was collected to quantify the obtained proteins with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). An equal amount (20 μg) of proteins was loaded and separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Merck Millipore, Burlington, MA). The membrane was blocked in 5% bovine serum albumin (BSA) for 2 h at room temperature and then incubated with primary antibodies (1;1000; anti-E protein, p-IRF3, and IRF3 antibodies) at 4 °C overnight. The membrane was then exposed to HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10,000) at room temperature for 2 h and the results visualized with an enhanced chemiluminescence kit (Super RX-N–C; Yestar Healthcare Holdings Company, Guangxi, China).

Plaque assay

A549 or Vero cells were seeded in 12-well plates (2×10^5 cells/well) with DMEM high glucose, 10% FBS, and 1% penicillin/streptomycin at 37 °C. When the cells were confluent, the medium was removed, and cells were washed with PBS. Then, viruses in DMEM supplemented with 1% FBS and 1% penicillin/streptomycin were added for 1 h. The unadsorbed viruses were washed off and cells were overlaid with 1 mL 1% agarose containing 2% FBS and 1% penicillin/streptomycin. The plates were cultured at 37 °C for 3 and 5 days for Vero and A549 cells, respectively. Finally, cells were stained with 1 mL 1% crystal violet solution in 10% methanol for 3 h, after which the stain was removed with tap water and the plaques were counted. The virus titter was expressed as plaque forming unites (PFU) per milliliter.

MTT Assay

Cells were seeded in 96-well plates (7,000–10,000 cells/well) and cultured at 37 °C for 24 h. A series of peptides at different concentrations were added into the medium and then cells were cultured at 37 °C for 48 h. Next, 20 μL MTT (Invitrogen, Carlsbad, CA) solution (5 mg/mL in PBS buffer) was added to each well and the plate was further incubated at 37 °C for 4 h. After removing the medium, 100 μL DMSO was added and the plate was incubated for 20 min at room temperature with shaking to completely dissolve the crystal purple formazan. Finally, absorbance was measured at 570 nm.

Peptide Antiviral Assay

As mentioned, cells were seeded in 12-well plates (2×10^5 cells/well) and cultured overnight, after which the medium was removed and the cells rinsed with PBS. Next, the cells were incubated with virus (multiplicity of infection [MOI] = 0.1; 2×10^4 PFU) and peptides in FBS-free DMEM. After 1 h of incubation, cells were rinsed and

replenished with medium containing peptides throughout the experiment. DENV RNA was then subjected to qRT-PCR after 48 h.

Viral Attachment Assay

A549 cells were incubated with virus (2×10^4 PFU) and peptides at 4 °C for 1 h and then rinsed with PBS and shifted to 37 °C for viral entry. After 1 h of incubation, cells were rinsed and replenished with fresh medium. DENV RNA was then subjected to qRT-PCR after 48 h.

Viral Entry Assay

Cells were infected with virus (2×10^4 PFU) for 1 h at 4 °C for viral attachment to cells, and then cells were rinsed before adding peptides. The incubation temperature was then shifted to 37 °C for viral entry. After 1 h of incubation, cells were rinsed and replenished with fresh medium. DENV RNA was then subjected to qRT-PCR after 48 h.

Viral Inactivation Assay

rSmp76 (40 μmol/L) was incubated with virus (2×10^4 PFU) for 1 h at 37 °C and then the mixture was diluted 50× and added to the cells for 1 h. After inoculation, the cells were rinsed with PBS and replenished with fresh medium. DENV RNA was then subjected to qRT-PCR after 48 h.

RNase Digestion Assay

Released genomic RNA from DENV was detected by RNase digestion assays and qRT-PCR as described by Yu *et al.* (2016). Briefly, DENV (2×10^4 PFU) was incubated with rSmp76 at room temperature for 1 h. Released genomic RNA from treated DENV was then digested with RNase A (D6950-01; Omega, Norcross, GA) at 37 °C for 1 h. After inactivation of residual RNase A, undigested genomic RNA in the intact viral particles was extracted using TRIzol reagent, reversed transcribed, and quantified as mentioned above.

Post-infection Assay

Cells were infected with virus (2×10^4 PFU), which was then removed after 1 h at 37 °C. The cells were rinsed and replenished with medium containing peptides throughout the experiment and then DENV RNA was subjected to qRT-PCR after 48 h.

Cell-Stimulation Assay

Cells were incubated with peptides for 1 h at 37 °C and then peptide-treated cells were rinsed before virus infection (2×10^4 PFU) for 1 h at 37 °C. Next, the cells were rinsed and replenished with fresh medium and then DENV RNA was subjected to qRT-PCR after 48 h.

qRT-PCR and Enzyme-Linked Immunosorbent Assay (ELISA) of IFN-β and Interferon-Stimulated Genes (ISGs)

Cells were seeded in 12-well plates at 2×10^5 cells/well. After overnight culturing, the medium was removed, and the cells were rinsed and replenished with FBS-free medium. Four different treatments were performed as follows, the mock and Smp76 groups were left uninfected, while the DENV and Smp76 + DENV groups were infected with DENV (MOI = 1; 2×10^5 PFU) for 1 h, after which the medium was removed and cells were rinsed with PBS. Mock and DENV groups were replenished with fresh medium, while Smp76 and Smp76 + DENV groups were replenished with fresh medium containing rSmp76 peptides. After 12 h, RNA and supernatants were harvested for qRT-PCR and ELISA, respectively. ELISA was performed using the VeriKine Human IFN βeta ELISA Kit (catalog no. 41410; PBL Assay Science, Piscataway, NJ) according to manufacturer's instructions.

Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and expressed as the mean ± standard deviation (SD) of at least three experiments.

Results

Prokaryotic Expression, Purification, and Identification of rSmp76

Scorpions are small arachnids that are mostly 5–6 cm in length and secrete a low amount of scorpion venom; a single scorpion specimen can normally produce 1–3 μL venom by mild electrical stimulation. It is thus difficult to isolate, purify, and identify the monomeric peptide component from natural scorpion venom. It is even more difficult to prepare a sufficient amount of scorpion venom peptide to investigate its biological functions and related mechanisms. Thus, developing a strategy for producing sufficient amounts of a single scorpion venom peptide is

very important. To address these shortcomings, we constructed a prokaryotic expression and purification system for the scorpion venom peptide Smp76, which was previously characterized from *Scorpio maurus palmatus*, by an integrated strategy of fusion proteins, affinity chromatography, and peptide release by enzymatic cleavage (Fig. 1B–1F). High-purity rSmp76 was prepared as described in the materials and methods section. SDS-PAGE and HPLC analysis of rSmp76 demonstrated that the collected component was a single peptide (Fig. 1C–1E), while MALDI-TOF-MS (Fig. 1F) revealed that the experimental molecular mass of the peptide containing three disulfide bonds (8397.24 Da) was similar to its theoretical molecular mass (8397.07 Da; Fig. 1A).

Smp76 Inhibits DENV/ZIKV RNA Infection and Protein Synthesis

Smp76 shares high sequence similarity with two scorpion venom peptides, scorpine and HS-1, that possess antibacterial activities against multiple microorganisms (Fig. 1A) (Carballar-Lejarazu *et al.* 2008; Uawonggul *et al.* 2014). In addition, scorpine exhibits the inhibitory activity to DENV (Carballar-Lejarazu *et al.* 2008). Thus, we tested the antiviral activity of rSmp76 and found that rSmp76 can inhibit DENV-2 (*TSV01*) RNA replication (Fig. 2A) and protein synthesis (Fig. 2B) in A549 cells in a dose-dependent manner. Inhibitory rates of DENV-2 (*TSV01*) infection were 10.4, 26.4, 44.1 and 75.7% by rSmp76 under concentrations 1, 2, 5 and 10 μmol/L, respectively. The IC_{50} value of rSmp76 against DENV-2 (*TSV01*) was 6.21 μmol/L in A549 cells. Viral infectious particles in the supernatant were also reduced significantly (Fig. 2C, Fig. 2D). Additionally, the more virulent DENV-2 strain *NGC* was also used to test the antiviral activity of rSmp76, which led to similar results as for DENV-2 strain *TSV01* (Fig. 2E–2H). Furthermore, DENV-2 (*TSV01*) infection was effectively inhibited by rSmp76 in other cells lines, including Huh7, THP-1, and primary mouse macrophages (Fig. 2I–2K), and the half maximal inhibitory concentrations (IC_{50}) for rSmp76 against DENV-2 (*TSV01*) were revealed to be 6.21, 6.52, 4.69, and 5.17 μmol/L in A549, Huh7, and THP-1 cells as well as primary mouse macrophages, respectively. Meanwhile, cellular infection with ZIKV (Fig. 2L–2N) and HCV (data not shown) were also suppressed by the rSmp76 peptide. The inhibitory rates of rSmp76 against ZIKV were 17.3%, 34.9%, 38.8% and 73.8%, respectively, under the concentrations of 1, 2, 5 and 10 μmol/L. The IC50 value of rSmp76 against ZIKV was 6.63 μmol/L (Fig. 2L–2N). In contrast, rSmp76 did not affect HSV-1 infection, even under different concentrations (Supplementary Fig. S1 A–C). Altogether, the scorpion venom peptide Smp76 was found to effectively inhibit cellular infection by various viruses under non-cytotoxic concentrations (Supplementary Fig. S2).

rSmp76 Plays Antiviral Roles at the Post-infection Stage of the Viral Life Cycle

rSmp76 inhibited DENV infection in multiple cell types (Fig. 2A, 2I–2K). To investigate the antiviral mechanism of rSmp76, we incubated the viruses and/or cells with rSmp76 at different stages of the viral life cycle (Fig. 3A) and analyzed its antiviral activity (Fig. 3B–3G). We found that rSmp76 did not inactivate viral particles directly (Fig. 3B), as it did not affect virus infectivity after co-incubating with the peptide before infecting cells with the virus. Furthermore, rSmp76 also did not inhibit virus attachment to cells (Fig. 3D) nor viral entry (Fig. 3E). Interestingly, rSmp76 exhibited significant antiviral activity when pre-incubated with cells before infection (Fig. 3C) and after establishment of DENV infections (Fig. 3F). Thus, our findings suggest that rSmp76 mainly plays antiviral roles at the post-infection stage of the viral life cycle.

rSmp76 Upregulates IFN-β by Activating IRF3 Phosphorylation

IFN-mediated antiviral responses are critical against viral infection and pathogenesis (Schoggins *et al.* 2011; Seth *et al.* 2006; Muller *et al.* 1994). Therefore, we examined whether the antiviral function of rSmp76 is associated with IFNs. The following IFNs screening revealed that rSmp76 antiviral activity was associated with IFN-β regulation (Fig. 4). When rSmp76 or IFN-β (1 ng/mL) were added to cells 1 h post-infection, both exhibited antiviral activity (Fig. 4A). In contrast, when rSmp76 or IFN-β (1 ng/mL) were added to cells 2 days post-infection, neither exhibited any antiviral activity (Fig. 4B). The consistency in antiviral activity between rSmp76 and IFN-β indicated a relationship. In subsequent experiments, we observed that rSmp76 promotes IFN-β expression at the transcriptional and translational levels in both uninfected and infected cells (Fig. 4C, 4D). When virus-infected cells were treated with rSmp76, IFN-β expression was upregulated in a dose-dependent manner (Fig. 4E). For further confirmation, we tested the antiviral activity of rSmp76 in peritoneal macrophages from IFN-α/β receptor-deficient (*Ifnar1*$^{-/-}$) and wild-type mice. Indeed, rSmp76 was found to inhibit DENV infection in peritoneal macrophages from wild-type mice (Fig. 4F) but not in those from *Ifnar*$^{-/-}$ mice (Fig. 4G), suggesting that the antiviral activity of rSmp76 relies on the type-I IFN system. rSmp76 was also found to upregulate IFN-β expression by enhancing IRF3 phosphorylation (Fig. 4H). Thus, we concluded that rSmp76

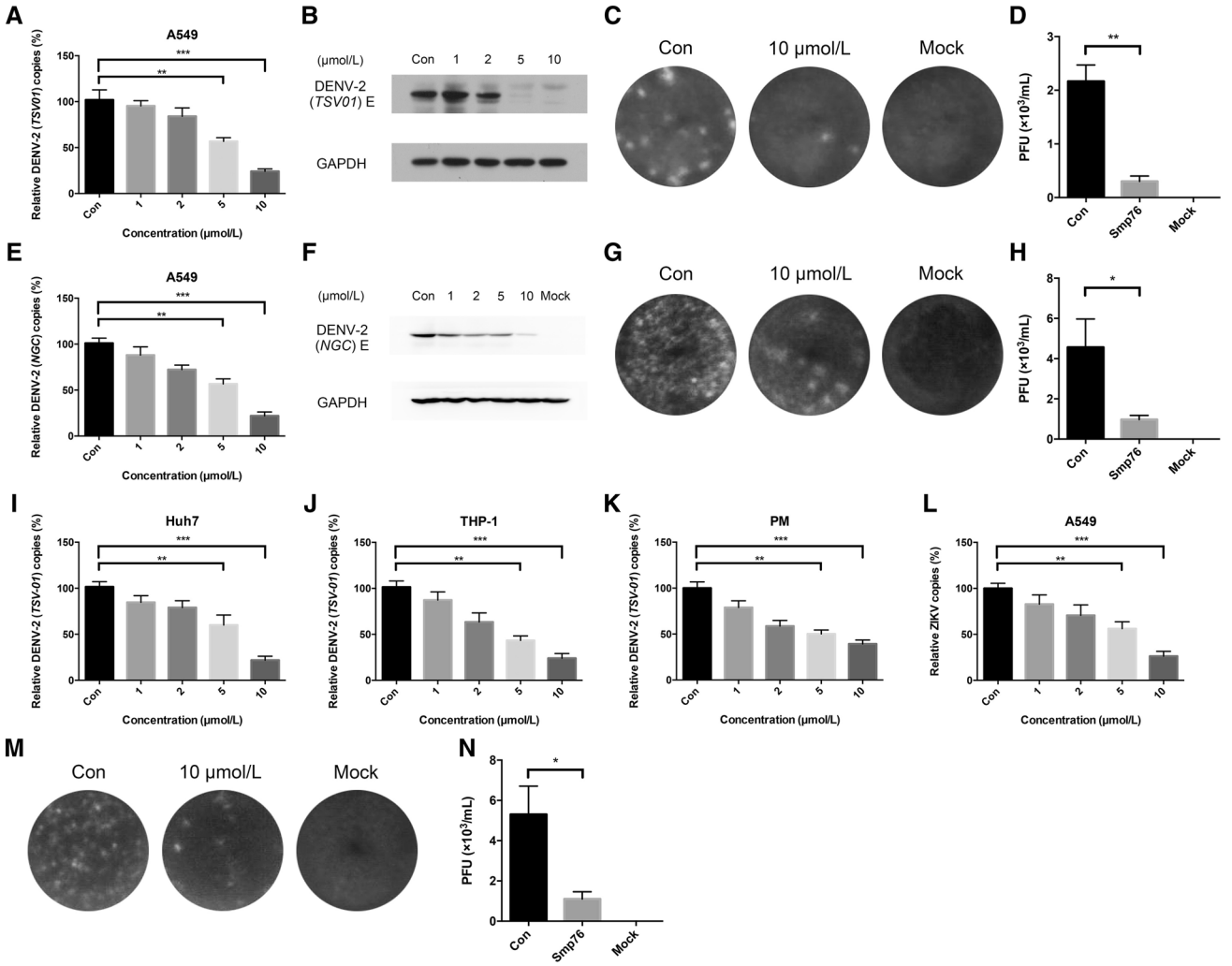

Fig. 2 Dose-dependent inhibitory activity of rSmp76 against DENV and ZIKV *in vitro*. **A**–**D** rSmp76 dose-dependent inhibition of DENV-2 (*TSV01*) infected-A549 cells. **E**–**H** rSmp76 dose-dependent inhibition of DENV-2 (*NGC*) infected-A549 cells. **A**, **E** RNA levels were analyzed by qRT-PCR. **B**, **F** Intracellular DENV-2 E protein levels were analyzed by western blotting. **C**, **D**, **G**, **H** Extracellular DENV-2 particles were diluted 100 × and quantified by plaque formation. **I**–**K** rSmp76 dose-dependent inhibition of DENV-2 (*TSV01*) infected-Huh7, THP-1, and mouse peritoneal macrophages (PM) analyzed by qRTPCR. **L** Dose-dependent inhibition of rSmp76 to ZIKV (PRVABC59) infection in A549 cells. (**M** and **N**) Plaque assay. Extracellular ZIKV (PRVABC59) particles were diluted 100 × and quantified by plaque formation. Data represent the mean ± SD of three independent experiments. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ (Student's *t*-test). Con, control. PM, mouse peritoneal macrophages. The internal controls of subfigure **A**, **E** and **I**–**L** are *GAPDH*.

inhibits DENV by enhancing IRF3 phosphorylation, which triggers IFN-β upregulation. Furthermore, it was previously reported that IFN-β does not inhibit HSV-1 infection efficiently *in vitro* as HSV-1 is not sensitive to IFN-β (Sainz and Halford 2002); this may partly explain why rSmp76 did not affect HSV-1 infection under our experimental conditions (Supplementary Fig. S1 A–C).

Effects of rSmp76 on Downstream ISGs

When IFN-α/β receptors receive stimulation from IFN-β, hundreds of cellular genes are upregulated, leading to antiviral responses (Jones *et al.* 2005; Morrison *et al.* 2013). ISG15 and OAS2 are the major downstream ISGs of IFN-β, and both possess anti-DENV functions (Hishiki *et al.* 2014; Dai *et al.* 2011; Lin *et al.* 2009). After exposing A549 cells to four different treatments (see "Materials and Methods" section), qRT-PCR was employed to detect ISG15 and OAS2 mRNA levels. As a result, ISG15 and OAS2 were found upregulated in response to rSmp76 treatment of both uninfected and infected cells (Fig. 5A, 5C). When cells were infected with DENV, ISG15 and OAS2 were also upregulated in a dose-dependent manner (Fig. 5B, 5D). However, ISG15 level of DENV-infected Smp76-treated cells was lower than that of uninfected Smp76-treated cells, which may be related to the fact that

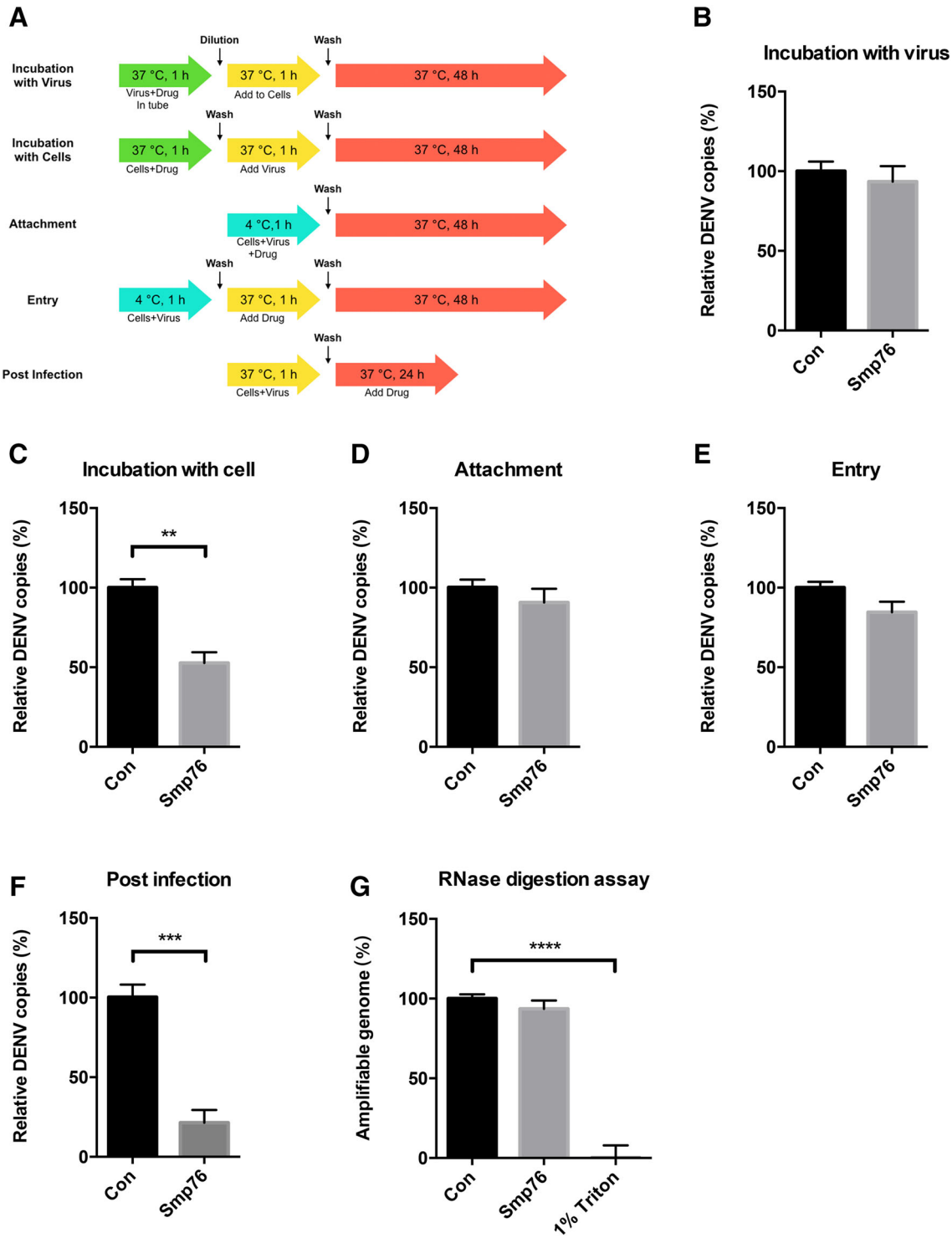

Fig. 3 Analysis of scorpion venom peptide rSmp76 effects during the viral life cycle. **A** Schematic diagram summarizing cells and/or virus treatment with rSmp76 (10 μmol/L). **B** Viral inactivation assay. **C** Cell stimulation assay. **D** Viral attachment assay. **E** Viral entry assay. **F** Post-infection assay. **G** RNase digestion assay. Data represent the mean ± SD of three independent experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$ (Student's *t*-test). Con, control. The internal controls of subfigure **B**–**F** are *GAPDH*.

DENV can degrade STAT2, downregulating transcription and translation of ISG15 (Morrison *et al.* 2013). Overall, our findings indicate that rSmp76 upregulates IFN-β, which increases expression levels of ISG15 and OAS2 and results in viral infection inhibition.

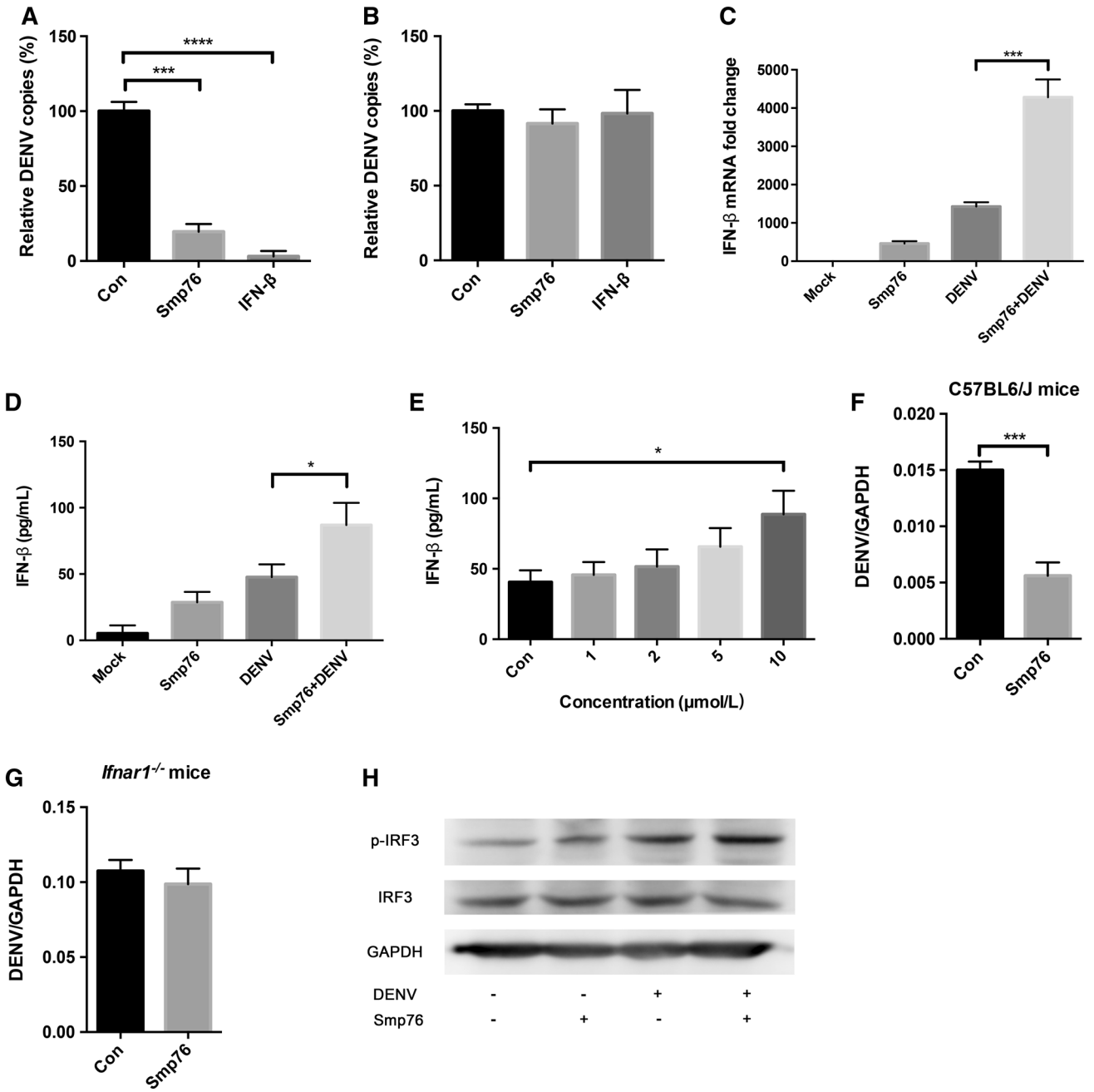

Fig. 4 rSmp76 upregulates IFN-β by activating IRF3 phosphorylation. **A** Comparison of the antiviral activity of rSmp76 (10 μmol/L) and IFN-β (1 ng/mL) against DENV at 1 h post-infection. **B** Comparison of the antiviral activity of rSmp76 (10 μmol/L) and IFN-β (1 ng/mL) against DENV at 48 h post-infection. **C** qRT-PCR expression levels of IFN-β. **D** ELISA of IFN-β protein levels in supernatants. **E** rSmp76 dose-dependent enhancement of IFN-β expression detected in the supernatant. **F** and **G** Antiviral effect of rSmp76 on peritoneal macrophages from wild type C57BL6/J (**F**) and *Ifnar1*$^{-/-}$ mice (**G**). DENV RNA levels were normalized to GAPDH. **H** Effect of rSmp76 on IRF3 phosphorylation by western blot analysis. The A549 cells were infected by DENV-2 (TSV01) with MOI = 1 for 12 h. Data represent the mean ± SD of three independent experiments. *$P \leq 0.05$; ***$P \leq 0.001$; ****$P \leq 0.0001$ (Student's *t*-test). Con, control. The internal controls of subfigure **A**, **B** and **C** are *GAPDH*.

Discussion

AMPs is an important self-protective factor against pathogenic microorganisms. In the present study, rSmp76 was found to inhibit DENV and ZIKV *in vitro* in a dose-dependent manner. Moreover, we found that Smp76 is not a virucidal peptide because it does not inactivate virus particles directly (Fig. 3B) or destroy viral envelope proteins tested by RNase digestion assays (Fig. 3G), but rather stimulates cells to upregulate IFN-β, achieving antiviral

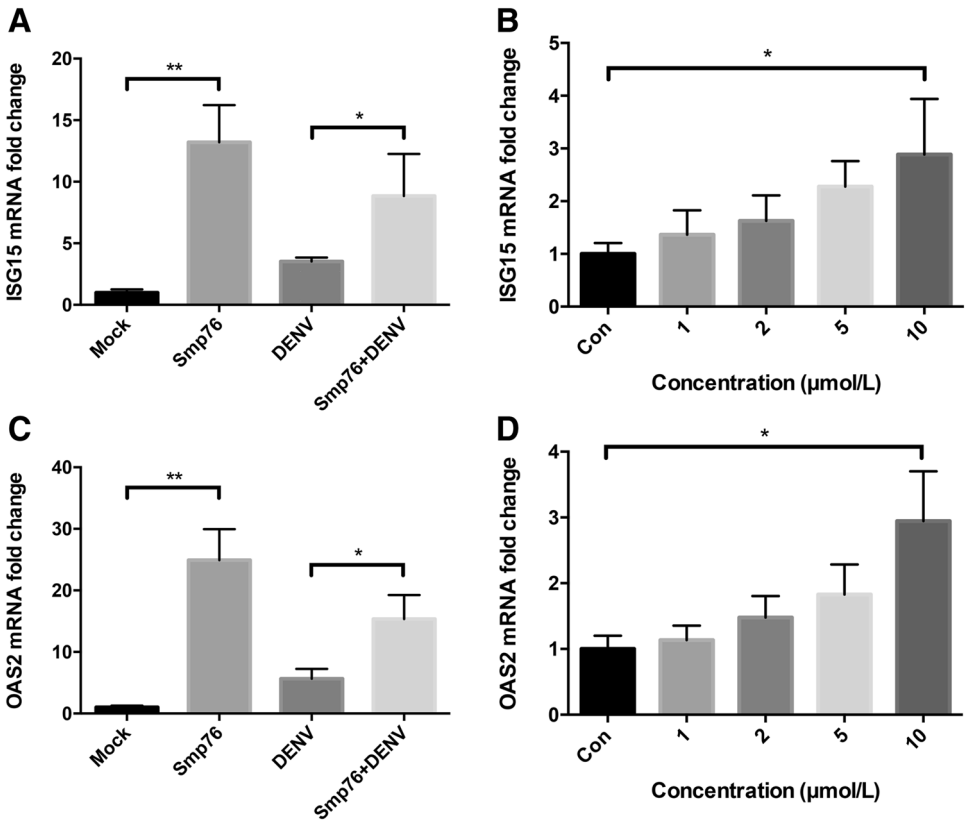

Fig. 5 rSmp76 upregulates ISGs downstream of the type-I IFN response. **A** qRT-PCR expression levels of ISG15. **B** Dose-dependent enhancement of ISG15 expression levels in DENV-infected cells treated with rSmp76. **C** qRT-PCR expression levels of OAS2. **D** Dose-dependent enhancement of OAS2 expression levels in DENV-infected cells treated with rSmp76. Data represent the mean ± SD of three independent experiments. $*P \leq 0.05$; $**P \leq 0.01$ (Student's *t*-test). Con, control. The internal controls of subfigure **A**–**D** are *GAPDH*.

function. In previous studies, most peptides, including natural (Carballar-Lejarazu *et al.* 2008; Holthausen *et al.* 2017; Hong *et al.* 2014; Jiang *et al.* 1993; Yasin *et al.* 2004) and synthetic (Hong *et al.* 2013; Schmidt *et al.* 2010; Yu *et al.* 2017b) peptides, were revealed to play antiviral roles by inactivating viral particles or inhibiting viral cellular entry. In addition to direct virucidal activity, some AMP members were also reported to possess immunomodulatory functions (Easton *et al.* 2009; Hancock and Finlay 2004; Hilchie *et al.* 2013; Scott *et al.* 2007). For example, the peptide Mucroporin-M1 can activate the MAPK pathway and downregulate HNF4α to inhibit HBV replication *in vitro* and *in vivo* (Zhao *et al.* 2012). In fact, the greatest difficulty during treatment of viral diseases is clearance of established infections. Our study demonstrated that the scorpion venom peptide Smp76 can upregulate IFN-β to suppress the established viral infections, which is a more potent and efficient way than traditional AMPs to treat viral infections.

Treatment of viral infections with direct-acting antivirals (DAAs) may exhibit decreased efficacy due to the high variability of viruses (Theofilopoulos *et al.* 2005). Therefore, an alternative treatment may enhance the protective effects of host innate immunity, such as IFN activation, against DENV and ZIKV progression. Such a strategy can potentially avoid drug resistance and the effects of genetic variability in the viral genome (Yu *et al.* 2017a). Numerous studies have demonstrated that type-I IFN is involved in the pathogenesis of DENV infection and the progression from undifferentiated febrile illness (DF) to life-threatening infection (Pichlmair and Reis e Sousa 2007; Ubol *et al.* 2008). Therefore, the Smp76-induced IFN response may function as a potential anti-DENV factor by exerting cytoprotective effects against DENV-associated cell damage and life-threatening symptoms associated with dengue hemorrhagic fever and dengue shock syndrome (Yu *et al.* 2017a).

Smp76, a scorpine-like peptide from the venom of the scorpion *Scorpio maurus palmatus*, was successfully expressed and purified in an *E. coli* BL21(DE3) system by an integrated strategy of fusion proteins, affinity chromatography, and enzymatic cleavage. Smp76 was found to inhibit DENV and ZIKV infections in cultured human cell lines and primary mouse macrophages in a concentration-dependent manner. Mechanistically, Smp76 does not inactivate viral particles directly but suppresses established viral infection by upregulating IFN-β expression through IRF3 phosphorylation, which enhances type-I IFN responses to inhibit viral infection. However, it is necessary to elucidate the detailed molecular mechanisms of Smp76 that promotes IFN-β expression during viral infection in future studies.

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Author Contributions FFL, ZQX, XCG and ZLJ designed the experiments and analyzed the data. ZLJ, FS and MJG performed most of the experiments. ZLJ and YTC wrote the manuscript. SAA, WXL, YLW and ZJC revised the manuscript. All authors read and approved the final manuscript.

Compliance with Ethics Standards

Conflict of interest The authors declare that they have no competing interests.

Animal and Human Rights Statement All animal experiments were in accordance with and were approved by the Institutional Animal Care and Use Committee of Wuhan University (Wuhan, China).

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