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Antiviral and anti-inflammatory activity of budesonide against human rhinovirus infection mediated via autophagy activation

Seong-Ryeol Kim[a,1], Jae-Hyoung Song[a,1], Jae-Hee Ahn[a], Geun-Shik Lee[b], Huijeong Ahn[b], Sung-il Yoon[c], Seung Goo Kang[c], Pyeung-Hyeun Kim[d], Sang-Min Jeon[e], Eun-Ji Choi[e], Sooyoung Shin[e], Younggil Cha[a], Sungchan Cho[f], Dong-eun Kim[f], Sun-Young Chang[e,**,2], Hyun-Jeong Ko[a,*,2]

[a] Laboratory of Microbiology and Immunology, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea
[b] College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Republic of Korea
[c] Division of Biomedical Convergence, School of Biomedical Science and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 24341, Republic of Korea
[d] Department of Molecular Bioscience, School of Biomedical Science and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 24341, Republic of Korea
[e] College of Pharmacy, Ajou University, Suwon, Republic of Korea
[f] Anticancer Agent Research Center, Korea Research Institute of Bioscience & Biotechnology, Ochang, Republic of Korea

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ABSTRACT

Human rhinovirus (HRV) infection causes more than 80% of all common colds and is associated with severe complications in patients with asthma and chronic obstructive pulmonary disease. To identify antiviral drug against HRV infection, we screened 800 FDA-approved drugs and found budesonide as one of the possible drug candidates. Budesonide is a corticosteroid, which is commonly used to prevent exacerbation of asthma and symptoms of common cold. Budesonide specifically protects host cells from cytotoxicity following HRV infection, which depend on the expression of glucocorticoid receptor. Intranasal administration of budesonide lowered the pulmonary HRV load and the levels of IL-1β cytokine leading to decreased lung inflammation. Budesonide regulates IL-1β production following HRV infection independent of inflammasome activation. Instead, budesonide induces mitochondrial reactive oxygen species followed by activation of autophagy. Further, the inhibition of autophagy following chloroquine or bafilomycin A1 treatment reduced the anti-viral effect of budesonide against HRV, suggesting that the antiviral activity of budesonide was mediated via autophagy. The results suggest that budesonide represents a promising antiviral and anti-inflammatory drug candidate for the treatment of human rhinovirus infection.

1. Introduction

Human rhinovirus (HRV) is a non-enveloped virus belonging to the *Picornaviridae* family. HRV consist of a viral capsid and a single strand of positive sense RNA (Puhakka et al., 1998). HRV is one of major causative agents of viral pulmonary infection, which triggers acute exacerbation of lower respiratory tract diseases including asthma and chronic obstructive pulmonary disease (COPD) (Johnston et al., 1995; Nicholson et al., 1993; Seemungal et al., 2000, 2001). Despite tremendous efforts to develop effective antiviral drugs for HRV infection, no treatment is currently available. Several medications have been used for the prevention of asthma exacerbation (O'Byrne et al., 2001; O'Byrne, 2011; Busse et al., 2001; Hanania et al., 2011). Budesonide is a corticosteroid, which can be used in combination with a long-acting beta agonist such as formoterol to prevent exacerbation of asthma and symptoms of common cold (Davies et al., 2011). Budesonide is a commonly used to treat children with asthma (Silverman and Otley, 2011) and is generally used as a nasal spray to treat allergic rhinitis (Wang and Zhang, 2015).

IL-1β is an important pro-inflammatory cytokine in host immunity.

* Corresponding author. Laboratory of Microbiology and Immunology, College of Pharmacy, Kangwon National University, Chuncheon 200-701, Republic of Korea.
** Corresponding author. Laboratory of Microbiology, College of Pharmacy, Ajou University, Suwon, Kyeonggi-do 443-749, Republic of Korea.
E-mail addresses: sychang@ajou.ac.kr (S.-Y. Chang), hjko@kangwon.ac.kr (H.-J. Ko).
[1] These authors contributed equally to this work.
[2] Co-senior author.

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However, excessive production of IL-1β also triggers acute lung injury and also exacerbates acute lung inflammation (Kolb et al., 2001). Transcription of pro-IL-1β is mediated via activation of NF-κB signaling (Bonizzi and Karin, 2004). The maturation and secretion of IL-1β is regulated by the inflammasome, in which activation of caspase 1 by several nucleotide-binding oligomerization domain-like receptors (NLRs) is critical for the IL-1β activation (Martinon et al., 2002). The levels of IL-1β were enhanced in the primary bronchial epithelial cells by the calcium flux-dependent activation of NLRP3 and NLRC5 inflammasome (Triantafilou et al., 2013).

In the current study, we showed that budesonide has antiviral activity against rhinovirus *in vitro* and *in vivo*, which correlates with the controlled IL-1β production in the lungs of HRV1B-infected mice after budesonide treatment. We found that budesonide enhanced the levels of mitochondrial reactive oxygen species (ROS), which in turn activated autophagy. Chemical inhibitors of autophagy attenuated the antiviral activity of budesonide *in vitro* and *in vivo*, suggesting that activation of autophagy pathway might be responsible for the antiviral activity of budesonide.

2. Materials and methods

2.1. Cell culture, viruses, and reagents

Human rhinovirus 1B (HRV1B), coxsackievirus B3 (CVB3), and enterovirus 71 (EV71) were obtained from ATCC (Manassas, VA, USA) and HRV1B was propagated by infection at 33 °C in HeLa cells, and CVB3 and EV71 were propagated by infection at 37 °C in Vero cells. HeLa cells and Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). Budesonide, rupintrivir, chloroquine diphosphate salt and bafilomycin A1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxycycline was purchased from Clontech (Mountain View, CA, USA). For Tet-on system, the GCR shRNA-transfected HeLa cells were treated with doxycycline (2 μg/mL) in culture media every 2 days for 7 days.

2.2. Mice and virus infection

Four-week-old, female BALB/c mice and C57BL/6 mice were purchased from SPL animal company (Orient Bio Inc, Sungnam, Korea), and IL-1R$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in accordance with the guidelines, and stabilized for 7 days at Kangwon National University. Mice were infected intranasally with HRV1B (1.8×10^7 pfu/mouse) three times with 10 μL/PBS at intervals of 10 min. Budesonide was intranasally administered with 0.1 mg/kg after HRV1B infection.

2.3. SRB assay

The SRB assay was used to measure the antiviral activity as previously reported (Song et al., 2014). The day before the experiment, HeLa and Vero cells (3×10^4 cells/well) were seeded in a 96-well culture plate. HeLa cells were used for HRV1B infection, and Vero cells were used for CVB3 and EV71. After 24 h, the HRV1B infection medium was replaced with 30 mM $MgCl_2$, 1% FBS in MEM media. The CVB3 and EV71 were replaced with a medium containing 1% FBS in MEM. The HRV1B-infected HeLa cells were incubated at 33 °C and 5% CO_2, and CVB3-, EV71-infected Vero cells were incubated at 37 °C and 5% CO_2, resulting in CPE. After 48 h, the 96-well culture plate was washed with PBS, and fixed in 70% acetone (100 μL/well) at 30 min, followed by staining with 0.4% SRB (Sigma-Aldrich, St, Louis, MO, USA) in 1% acetic acid. The precipitated SRB crystals were solubilized with 10 mM unbuffered tris-based solution 100 μL/well. The absorbance was read on a SpectraMax i3 microplate reader (Molecular Devices, Palo Alto, CA, USA) at 562 nm.

2.4. Real-time PCR

Total RNA was extracted from HeLa cells and mice lung lysate with a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed with RNase inhibitor, oligo (dT) 15 primers, dNTP mixture, and Moloney murine leukemia virus reverse transcriptase with 5× buffer, according to the established protocol (Promega, Madison, WI, USA). Quantitative real-time PCR (qPCR) analysis was performed to amplify complementary deoxyribonucleic acid (cDNA), using the THUNDERBIRD® SYBR® qPCR mix (Toyobo, Osaka, Japan), and CFX96 optics module real-time PCR system (Bio-Rad, Hercules, CA, USA). We used the following primers: HRV 5′-NCR sense, 5′-TCC TCC GGC CCC TGA ATG-3′ and HRV 5′-NCR-antisense, 5′-GAA ACA CGG ACA CCC AAA G-3′; and human β-actin-sense, 5′-CCA TCA TGA AGT GTG ACG TGG-3′ and human β-actin-antisense, 5′-GTC CGC CTA GAA GCA TTT GCG-3′. The PCR conditions were as follows: 95 °C for 3 min for 1 cycle and 95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; for 35 cycles.

2.5. Cytokine and chemokine assays

The cytokine levels were evaluated in the supernatants of HRV1B-infected mouse lung lysate using the IL-1β and IL-6 ELISA kits (eBioscience, San Diego, CA, USA). The lung tissue was homogenized in CK28 (Bertin Technology, Orsay, France) using 2.8-mm ceramic beads with 600 μL PBS, and using a Minilys homogenizer (Bertin Technology) at 6000 rpm, for 15 s, twice at −20 °C. The clear supernatants were collected following centrifugation at 10,000*g* for 10 min, at 4 °C (Song et al., 2017). The absorbance was read at 450 nm using a SpectraMax 340 (Molecular Devices).

2.6. Histological analysis

The HRV1B-infected lungs of mice were washed in PBS, and fixed with 4% (w/v) formaldehyde overnight. The HRV1B-infected lungs were dehydrated in serial gradients of ethanol and xylene, and embedded in paraffin. The tissues were sliced into 5-μm-thick sections and stained with hematoxylin and eosin. After HRV1B infection, the lungs with severe inflammation (score: 0–4) were graded for inflammation, edema, and cellular infiltration, according to the guidelines. A pathologist evaluated the degree of inflammation microscopically (200×) and was blinded to the scores. The means of lung inflammatory score were graded for severity (absent, minimal, mild, moderate, marked).

2.7. Western blot

SDS-PAGE was carried out as described previously (Hong et al., 2017). Protein levels were evaluated in the lysate of HRV1B-infected HeLa cells with primary antibodies including: LC3B:2775S, AMPK and ACC Antibody Sampler Kit: 9957 and α-tubulin: 2144 (Cell Signaling Technologies, Denver, MA, USA), SQSTM1 monoclonal antibody (p62): H00008878-M01 and anti-Glucocorticoid Receptor antibody: GTX101120 (GeneTex, Inc, Irvine, CA, USA), anti-rabbit Cytoskeletal Actin Antibody: A300-491A (Bethyl Laboratories, Montgomery, TX, USA), anti-caspase-1 p20 antibody: 06-503 (EMD Millipore, Burlington, MA, USA), anti-caspase-1 antibody: sc-622 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-NLRP3 antibodies: #AG-20B-0014-C100 (AdipoGen, San diego, CA, USA), anti-mouse IL-1β antibody: AF-401-NA (R&D Systems, Minneapolis, MN, USA), and secondary antibodies including: anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technologies), and goat anti-mouse IgG F(ab′)2, polyclonal antibody (HRP conjugate) (Enzo Life Sciences., Farmingdale, NY, USA). The elevated chemi-luminescence substrate used was femtoLUCENT™ PLUS HRP Kit (G-biosciences, St. Luise, MO, USA). Images were obtained with ImageQuant™ LAS 4000 mini system (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and analyzed using

Image J software (NIH, Bethesda, MD, USA).

2.8. Measurement of intracellular ROS

The day before the experiment, the HeLa cells (1.5×10^5 cells/well) were seeded in a 24-well culture plate. After 24 h, the cells were infected with HRV1B and treated with 1, 5 and 15 μM budesonide for 1 h in a medium containing 30 mM $MgCl_2$, and 1% FBS in MEM. Cells were washed with PBS, and treated with 10 μM MitoSOX (Invitrogen) and incubated for 30 min at 37 °C and 5% CO_2. Cells were washed with PBS, and fixed with 4% paraformaldehyde. Cells were analyzed using FACSVerse (BD Bioscience, San Diego, CA, USA) and the data were analyzed using BD FACSuite software application.

2.9. Confocal microscopy

HeLa cells (1×10^5 cells/well) were seeded in a 6-well culture plate. After 24 h, cells were infected with HRV1B and treated with 125 μM budesonide for 8 h, in a medium containing 30 mM $MgCl_2$, 1% FBS in MEM. Cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were stained with the primary antibodies including anti-LC3 Abs (Cell Signaling Technologies) and incubated overnight at 4 °C. After washing with PBS, the cells were stained with the secondary antibodies including goat anti-rabbit IgG H&L (Alexa Fluor® 647) (Abcam, Cambridge, MA, USA) and incubated for 2 h at room temperature. After washing with PBS, cells were stained with DAPI (4′, 6-diamidino-2-phenylindole) and analyzed using confocal microscopy (SM880 with Airycan, Zeiss, NY, USA).

2.10. Plasmid cloning and lentivirus production

Plasmid cloning and lentivirus production were carried out as described previously (Choi et al., 2017). To establish the Tet-inducible lentiviral shRNA expression system, all the shRNA oligo was annealed and inserted into the Tet-pLKO-puro vector, which was gifted by Dmitri Wiederschain (plasmid #21915, Addgene, Cambridge, MA, USA). We constructed the Tet-pLKOblast vector with the blasticidin resistance gene. For shRNA sequences, we used TRCN0000245004 for GCRsh-1. For lentivirus production in 293T cells, each lentiviral vector was co-transfected with pMD2.G and psPAX2 (gifted from Didler Trono: Addgene plasmid #12259, #12260), using Lipofectamine 2000.

2.11. Inflammasome activation

Priming: The day before the experiment, BMDMs (1×10^6 cells/well) were seeded in a 24-well culture plate and primed with HRV1B at an MOI of 10 or 10 μg/mL LPS (Sigma-Aldrich) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solutions for 3 h. After LPS priming, each well was treated with 1 μM budesonide for 1 h. Cell lysates (Lys) were collected for further analysis.

Activation: The day before the experiment, BMDMs (1×10^6 cells/well) were seeded in a 24-well culture plate and primed with 10 μg/mL LPS in an RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solutions for 3 h, followed by inoculation with HRV1B at MOIs of 1, 5, and 10 for 6 h. Cellular supernatant (Sup) and cell lysates (Lys) were collected for further analysis.

2.12. Statistical analyses

We compared multiple groups using a one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ were considered significant at a 95% confidence interval.

Table 1
Inhibition of human rhinovirus-mediated cytotoxicity by FDA-approved drugs.

Compound	HRV14			HRV15		
	CC_{50}[a]	IC_{50}[b]	TI[c]	CC_{50}[a]	IC_{50}[b]	TI[c]
Anti-cancer						
Temsirolimus	> 25	8.4 ± 0.7	2.97	> 25	5.5 ± 1.6	4.55
Clomiphene Citrate	> 25	1.9 ± 0.1	13.2	> 25	1.4 ± 0.1	17.9
Gemcitabine·HCl	> 25	0.8 ± 0.2	31.3	> 25	0.5 ± 0.1	50
Tamoxifen Citrate	> 25	4.8 ± 0.4	5.21	> 25	5.6 ± 1.0	4.46
Steroids						
Budesonide	> 25	4.3 ± 0.6	5.81	> 25	1.5 ± 0.4	16.7
Dexamethasone	> 25	3.1 ± 13	8.06	> 25	0.7	35.7
Anti-fungals						
Ketoconazole	> 25	7.8 ± 0.3	3.21	> 25	4.7 ± 1.1	5.32
Itraconazole	> 25	1.2 ± 0.1	20.8	> 25	0.4	62.5
Posaconazole	> 25	4.3 ± 0.4	5.81	> 25	0.5 ± 0.1	50
Terconazole	> 25	1.9 ± 0.2	13.2	> 25	1.1 ± 0.4	22.7
Micafungin	> 25	9.2 ± 0.3	2.72	> 25	4.8 ± 0.5	5.21

Results are presented as the mean IC_{50} values obtained from three independent experiments carried out in triplicate ± S.D.

[a] Concentration required to reduce cell growth by 50% (μM).

[b] Concentration required to inhibit virus-induced CPE by 50% (μM).

[c] Therapeutic index = CC_{50}/IC_{50}.

3. Results

3.1. Budesonide showed antiviral activity against human rhinovirus

We screened the FDA-approved drug library to develop a new antiviral drug against human rhinovirus from the currently available drugs using HeLa cell-based assay system. We determined the cytopathic effect of human rhinovirus at 48 h following viral infection after treatment with compounds at 25, 5, and 1 μM concentrations to evaluate the antiviral activity of candidate drugs. We identified several compounds exhibiting significant inhibition of human rhinovirus-mediated cytotoxicity in a dose-dependent manner (Table 1). Antifungals including itraconazole and anti-neoplastics such as gemcitabine show anti-viral activity against HRV by blocking cholesterol transport (Shim et al., 2016) or inhibiting the viral RNA synthesis (Song et al., 2017).

Other effective compounds include glucocorticoids such as budesonide and dexamethasone. Budesonide (1 μM) significantly blocked the HRV14-mediated cytotoxicity and the estimated IC_{50} of budesonide for HRV14-infected HeLa cells was about 4.3 ± 0.6 μM. Budesonide also inhibited the cytotoxicity of HeLa cells infected with HRV1B and HRV15 (Fig. 1A and Table 1). HRV1B viral replication was significantly reduced by budesonide treatment (Fig. 1B). Similar inhibitory effect of budesonide was observed in HeLa cells containing HRV14 replicon system although the anti-viral efficacy was lower than that of rupintrivir (Fig. 1C) at similar concentrations. The estimated IC_{50} of budesonide for the replication of HRV14 replicon was about 2.62 ± 1.01 μM.

The antiviral activity of budesonide was further assessed in EV71- or CVB3-infected Vero cells. However, we failed to detect any significant increase in viable EV71- or CVB3-infected Vero cells after treatment with budesonide, suggesting that the antiviral activity of budesonide was specific to human rhinovirus infection (Fig. 1D and E).

To assess whether the antiviral activity of budesonide was mediated via the glucocorticoid receptor (GCR), we conditionally depleted GCR in HeLa cells using the Tet-inducible knock out system. Treatment of GCR shRNA-transfected HeLa cells with doxycycline abolished the expression of GCR (Fig. 1F). The antiviral activity of budesonide against HRV1B was significantly reduced in GCR knock-down HeLa cells (Fig. 1G), suggesting that the antiviral activity of budesonide was

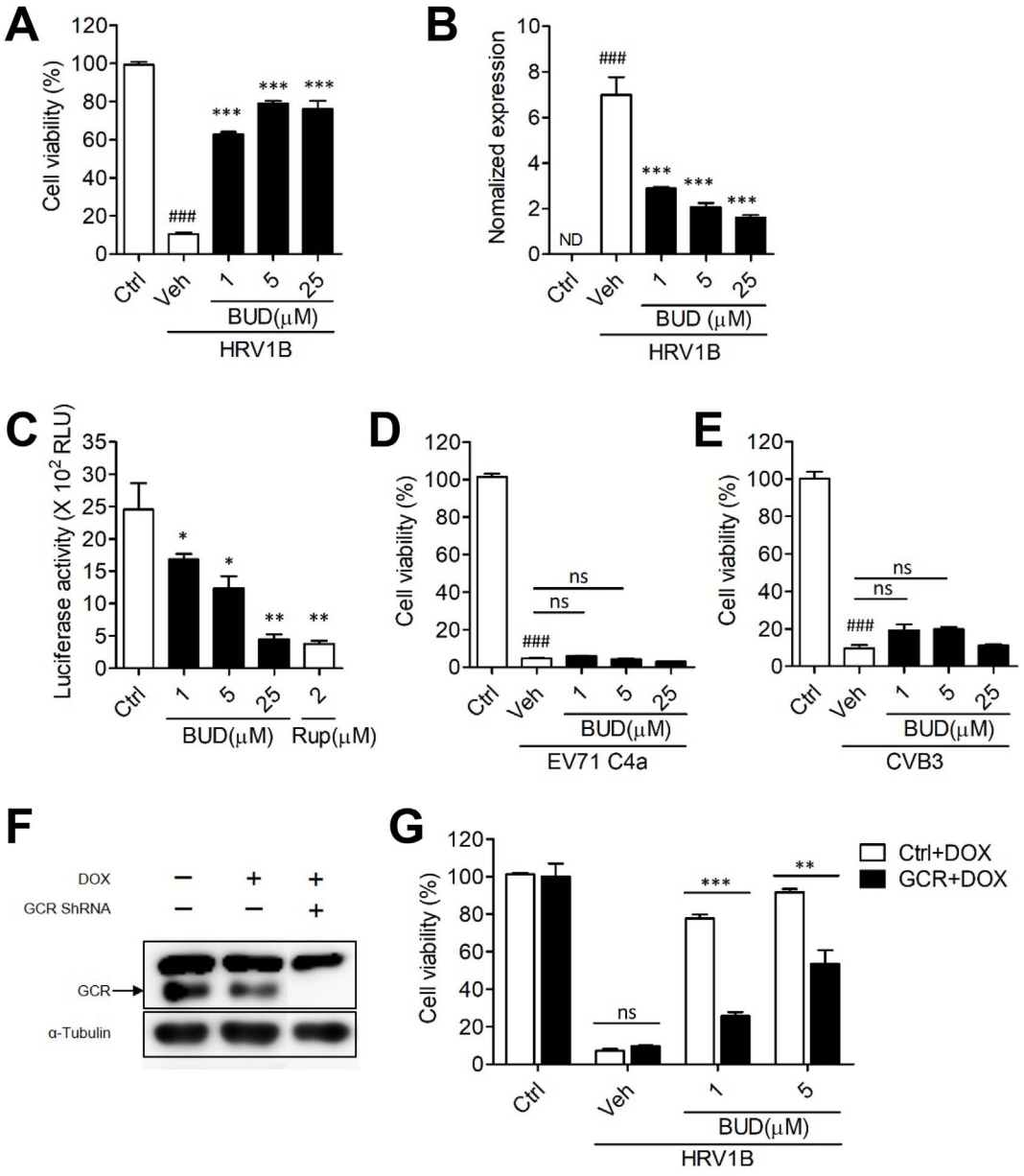

Fig. 1. The antiviral activity of budesonide against HRV1B *in vitro*. (A) The antiviral activities of budesonide (BUD) against HRV1B were determined by inoculating HeLa cells with HRV1B at an MOI of 10 and treated with budesonide. The viability of cells was measured using SRB assay, and the antiviral activity was calculated based on cell viability. Results are shown as means ± SEM. $^{###}p < 0.001$ for comparison with non-infected control group (Ctrl); $^{***}P < 0.001$ for comparison with HRV1B-infected vehicle group (Veh); (B) Relative HRV1B gene expression in HRV1B-infected HeLa cells was determined by real-time PCR. ND, not detected; $^{###}p < 0.001$ for comparison with control; $^{***}P < 0.001$ for comparison with vehicle; (C) *In vitro*-transcribed HRV14 replicon RNA was transfected into 293T cells, and the transfected cells were treated with the indicated concentrations of budesonide or rupintrivir (Rup) for 8 h. The relative luciferase activity (RLU) is shown. $^{*}P < 0.05$ and $^{**}P < 0.01$ for comparisons with control (Ctrl); (D–E) The amelioration of cytotoxicity by budesonide in EV71- or CVB3-infectd Vero cells was assessed. ns, not significant; $^{###}p < 0.001$ for comparison with control; (F) Knock-down of glucocorticoid receptor (GCR) was confirmed in GCR shRNA-transfected HeLa cells after treatment with 2 μg/mL doxycycline (DOX) for 7 days. Whole-cell lysates were collected for Western blotting analysis of GCR; (G) GCR shRNA-transfected HeLa cells were treated with 2 μg/mL doxycycline for 7 days and infected with HRV1B at an MOI of 10 in the presence of budesonide.

mediated by the GCR.

3.2. Budesonide inhibits HRV1B replication in vivo and ameliorates pulmonary inflammation

We next assessed whether budesonide elicited antiviral activity *in vivo*. Groups of mice were intranasally inoculated with HRV1B, and budesonide were also administered intranasally 10 min after the final infection. The lungs of mice were obtained at 8 h post-infection and assessed for the virus titer and pro-inflammatory cytokines including IL-1β and IL-6. Budesonide treatment completely eliminated HRV1B from murine lungs at 8 h after infection when confirmed by the gene expression of HRV1B non-coding region (NCR) (Fig. 2A). As expected, the levels of IL-1β and IL-6 were significantly increased in the HRV1B-infected lungs (Fig. 2B and C). Intriguingly, budesonide treatment significantly decreased the levels of IL-1β but not IL-6.

3.3. Partial blockade of IL-1β inhibits HRV1B infection, but complete ablation of IL-1R signaling exacerbated the viral infection

Although IL-1β is a crucial cytokine to protect the host against rhinovirus infection, the excessive and non-specific secretion of IL-1β in lung tissue might be detrimental. Therefore, we decided to investigate whether IL-1β blockade interfered with HRV replication. To this end,

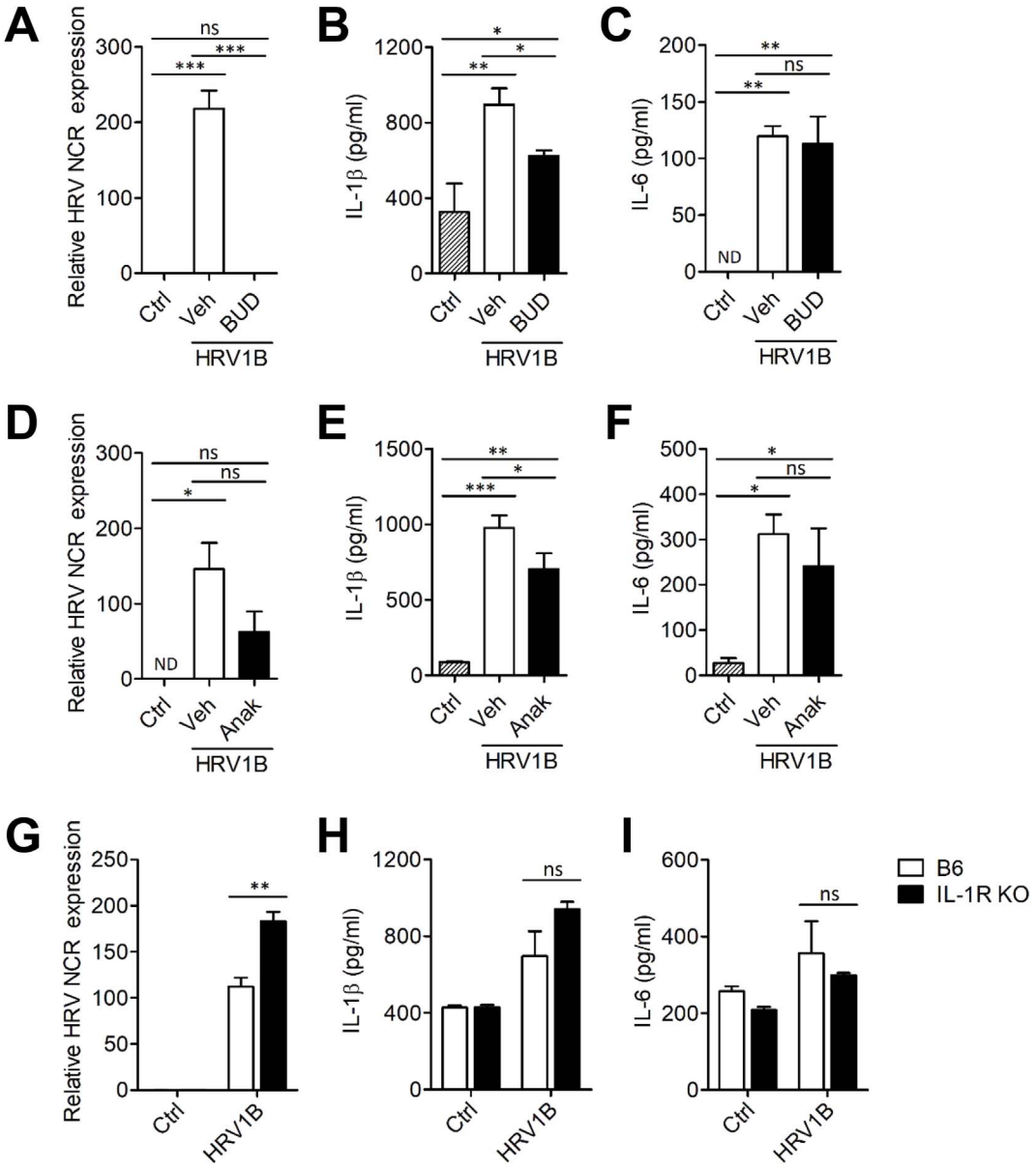

Fig. 2. Antiviral activity of budesonide in the lungs of HRV1B-infected mice with the reduced levels of IL-1β. (A–C) HRV1B-infected BALB/c mice were administered 0.1 mg/kg budesonide intranasally. (A) The relative HRV non-coding region (NCR) expression, (B) the production of IL-1β and (C) IL-6 were analyzed in lung tissue homogenates. (D–F) BALB/c mice were treated with 5 mg/kg anakinra (Anak) intranasally and infected with HRV1B. (D) The relative HRV NCR expression, (E) the expression of IL-1β, and (F) IL-6 were assessed in the lung tissue homogenates. (G–I) IL-1R knock-out (KO) mice were infected with HRV1B. (G) The relative HRV NCR expression, (B) IL-1β, and (C) IL-6 were analyzed in lung tissue homogenates. Results are shown as means ± SEM. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

we treated HRV1B-infected mice with Anakinra, an IL-1R antagonist, to inhibit IL-1β-mediated signaling. Interestingly, Anakinra treatment reduced viral replication reflected by the HRV1B NCR gene expression (Fig. 2D) and also decreased the level of IL-1β (Fig. 2E), but not IL-6 (Fig. 2F) in lung tissue.

To further confirm the role of IL-1β in HRV1B infection, we infected IL-1R knockout mice with HRV1B. In mice totally deficient in IL-1β signaling, the HRV1B replication was accelerated (Fig. 2G), without altering the secretion of IL-1β and IL-6 (Fig. 2H and I). Collectively, these results suggest that HRV1B infection induced IL-1β production. Regulation of IL-1β production induced by HRV protected the host airway system, however, the absence of IL-1R signaling abrogated host immune defense and exacerbated viral replication and lung inflammation in HRV infection.

3.4. Budesonide reduced the HRV1B-induced synthesis of pro-IL-1β

A previous study reported that HRV infection induced the activation of inflammasome in bronchial cells (Triantafilou et al., 2013). We next assessed whether the HRV infection triggered inflammasome activation in macrophages, which are the major cell types of IL-1β production. Incubation of bone marrow-derived macrophages (BMDM) with HRV1B for 3 h dramatically induced NLRP3 and pro-IL-1β similar to LPS treatment (Fig. 3A). Interestingly, budesonide treatment reduced the levels of pro-IL-1β, without inhibiting NLRP3. To further assess the role of inflammasome activation in increased IL-1β production after HRV1B infection, we investigated caspase-1 cleavage. BMDM were infected with HRV1B in the presence or absence of LPS and the cleavage of caspase-1 was determined in the supernatant and cell lysates. Procaspase-1 was enhanced by HRV1B infection in the BMDM (Fig. 3B). However, the cleaved caspase-1 was detected only in the supernatant of

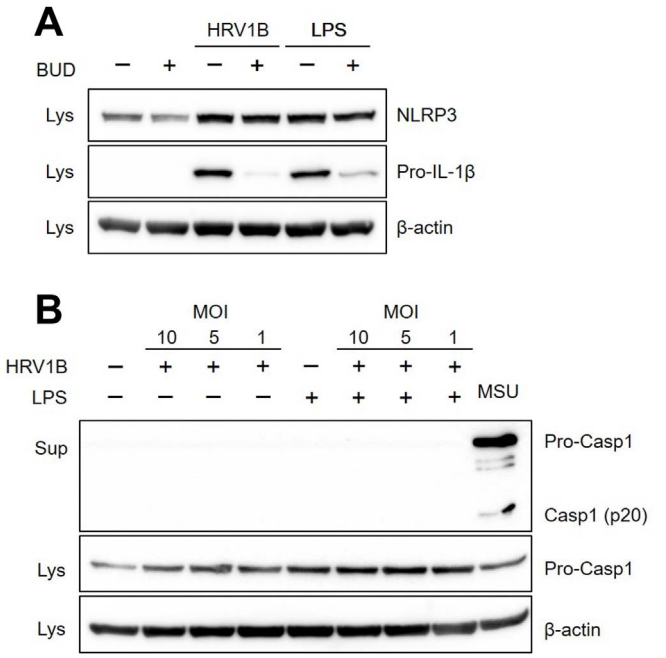

Fig. 3. Budesonide decreased the expression of pro-IL-1β by HRV1B infection. (A) Mouse bone marrow-derived macrophages (BMDMs) were primed with HRV1B at an MOI of 10 or 10 μg/mL LPS for 3 h, and treated with 1 μM budesonide. The expression of NLRP3, pro-IL-1β, and β-actin was analyzed in the cell lysate of BMDM. (B) Mouse BMDMs were primed with 10 μg/mL LPS for 3 h, and infected with HRV1B at MOIs of 10, 5, and 1 or treated with monosodium urate (MSU, 500 μg/mL) for 6 h. The levels of pro-caspase 1 (Pro-casp1) and cleaved caspase-1 (Casp1 p20) in culture supernatant of BMDM and pro-caspase 1 in cell lysate of BMDM were determined by Western blot.

BMDM treated with monosodium urate (MSU), which induces NLRP3 inflammasome, a caspase-1-activating complex. These results indicated that the decreased IL-1β production by budesonide after HRV infection might not be due to the inhibition of caspase-1 cleavage.

3.5. Budesonide enhanced autophagy induced by viral infection

The expression of pro-IL-1β and NLRP3 genes was induced by a NF-κB-dependent pathway. Reduction in NF-κB levels following budesonide treatment might be attributed to the reduction of IL-1β synthesis. However, the reduction of pro-IL-1β expression by budesonide may be induced via regulatory mechanisms other than NF-κB. It was recently shown that the inflammation-induced activation of autophagy increased the destruction of inflammasome component to inhibit IL-1β production (Shi et al., 2012). Thus, we assessed whether HRV1B infection induced autophagy activation. HRV1B infection of HeLa cells induced a slight increase in the conversion of LC3-I to LC3-II, which appeared to further increase following budesonide treatment (Fig. 4A). Budesonide treatment without HRV1B infection significantly induced the conversion of LC3-I to LC3-II. The degradation of P62 was increased by budesonide treatment. HRV1B infection also increased LC3 puncta formation in HeLa cells, which was further increased by budesonide treatment (Fig. 4B). The numbers of LC3 puncta were increased by budesonide treatment alone (Fig. 4C) consistent with LC3-II conversion and p62 degradation (Fig. 4A). These results suggested that HRV1B infection slightly increased autophagy formation, and budesonide treatment further activated the autophagy.

3.6. Budesonide increased mitochondrial ROS

To elucidate the mechanism of autophagy activation by budesonide, we first investigated the 5′ adenosine monophosphate-activated protein kinase (AMPK)-dependent phosphorylation of acetyl-CoA carboxylase (ACC), and mechanistic target of rapamycin (mTOR)-dependent phosphorylation of p70S6 kinase (P70S6K). Budesonide did not alter the phosphorylation level of ACC and P70S6K (Fig. 5A) suggested that AMPK and mTOR pathway had no role in autophagy activation by budesonide.

Autophagy can be initiated by reactive oxygen species (ROS) to clear all the irreversibly oxidized biomolecules including proteins, DNA and lipids within the cells (Filomeni et al., 2015). Treatment of HeLa cells with budesonide significantly increased the mitochondrial ROS level in a dose-dependent manner (Fig. 5B and C). Interestingly, budesonide treatment alone induced mitochondrial ROS. These results suggested that increased mitochondrial ROS mediated autophagy activation by budesonide.

3.7. Budesonide inhibits HRV infection via activation of autophagy

To investigate whether autophagy activation by budesonide played a critical role in the antiviral activity of budesonide against HRV1B infection, autophagy was blocked with inhibitors including chloroquine or bafilomycin A1. Both chloroquine and bafilomycin A1 significantly attenuated the protective activity of budesonide against HRV1B-mediated cytotoxicity (Fig. 6A). We also assessed whether chloroquine treatment inhibited the antiviral activity of budesonide in HRV1B-infected mice. We found that the mild-to-moderate inflammation induced by HRV1B infection including infiltration of inflammatory cells with mild edema and hemorrhage, was ameliorated by budesonide treatment. Conversely, it was significantly inhibited by chloroquine treatment (Fig. 6B and C). Interestingly, the IL-1β secretion in the lungs of HRV1B-infected mice having budesonide treatment was increased by chloroquine (Fig. 6D). Collectively, these results suggest that autophagy activation by budesonide inhibited both inflammation and IL-1β production, which might be attributed to the antiviral and anti-inflammatory effects of budesonide.

4. Discussion

Budesonide is an effective inhibitor of IL-1β transcription and is known to reduce excessive mucus secretions in asthma and respiratory inflammation (Proud et al., 1994). Corticosteroids act on the glucocorticoid receptors (GCR) in the cytoplasm. The steroid/GCR complex in the cytosol is translocated to the nucleus for binding to the glucocorticoid response element (GRE) (Blyth et al., 1998; Proud et al., 1994). Inhaled corticosteroids (ICSs) also result in synergistic effects when used in combination with a long-acting β2 agonist (LABA) by enhancing the bronchodilator response and increasing the expression of β2 adrenergic receptors (Calverley et al., 2003a; Pauwels et al., 1997; O'Byrne et al., 2005). Therefore, the dual therapy based on a combination of ICS and LABA has been widely used to control both asthma and COPD with clinical efficacy in decreasing the frequency of acute respiratory exacerbations in both disease states (Wells et al., 2012; Chung et al., 2009; Calverley et al., 2003b; Calverley et al., 2007). In patients with chronic disease states, acute exacerbations are a primary cause of morbidity. Therefore, research efforts have been directed at identifying therapeutic options to prevent HRV infection-induced acute attacks in both asthma and COPD patients. Previous studies reported that LABA plus corticosteroid therapy suppressed HRV replication *in vitro* (Yamaya et al., 2014; Bochkov et al., 2013; Skevaki et al., 2009). Findings from clinical studies also suggested that ICS therapy, when used alone or in combination with a LABA, prevented exacerbations of symptoms associated with common cold, particularly during the cold season (Bateman et al., 2011; Reddel et al., 2011). However, the effects of glucocorticoids on the HRV-induced inflammatory response in respiratory tract cells remain uncertain and merit further investigation. The present study explored the effects of budesonide on HRV-associated airway inflammation and viral replication.

HRV infection increased IL-1β and IL-6 in the lungs of infected mice.

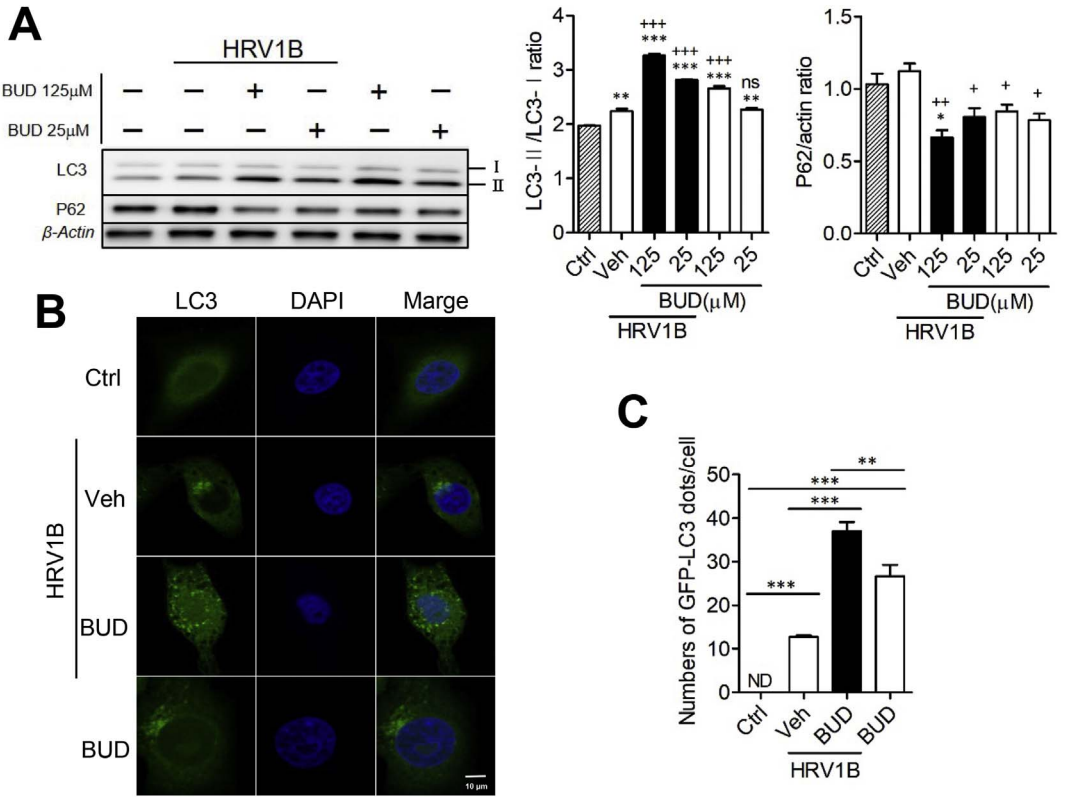

Fig. 4. Autophagy pathway was activated by budesonide in HRV1B-infected HeLa cells. (A) Western blotting analysis of LC3 and p62 in HRV1B-infected and non-infected HeLa cells after treatment with budesonide (25 μM and 125 μM) for 8 h. The ratio of LC3-II/LC3-I and the expression levels of P62 normalized with β-actin are shown in the right panel. Results shown are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for comparisons with non-infected control (Ctrl), $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ for comparisons with HRV1B-infected vehicle (Veh). (B) Representative image of LC3 staining (green fluorescence) in HRV1B-infected and non-infected HeLa cells treated with 125 μM budesonide for 8 h. DAPI straining of nucleus. (C) The numbers of GFP-LC3 dots per cell were counted. Results are shown as means ± SEM. ND, not detected, ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$.

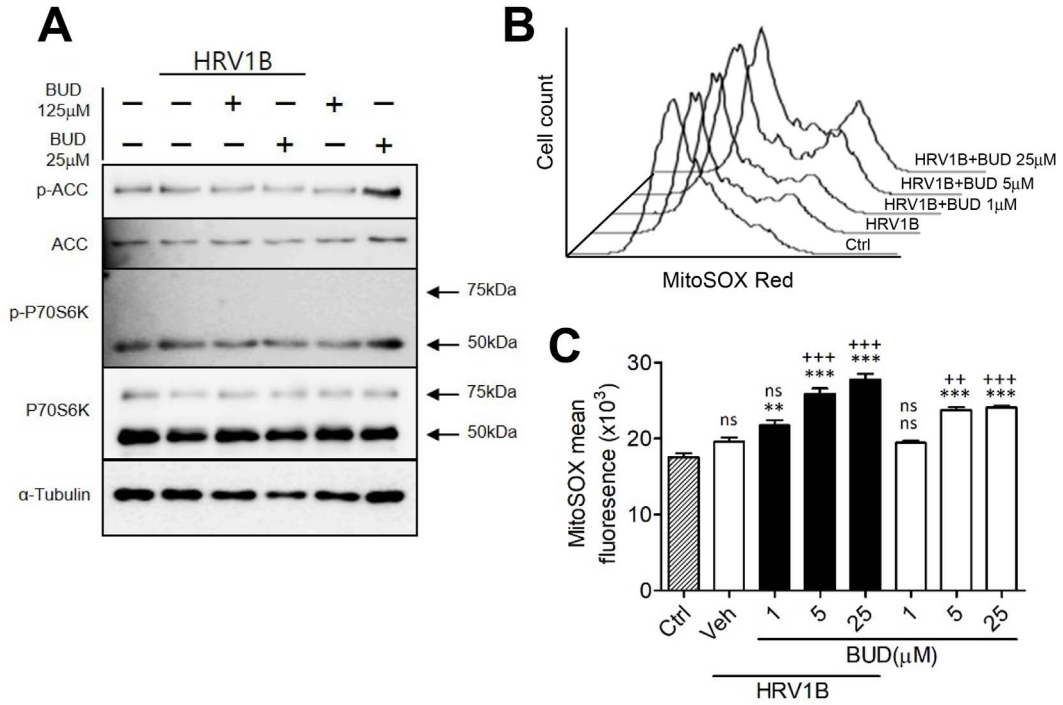

Fig. 5. Budesonide induced mitochondrial ROS. (A) Western blotting analysis of p-ACC, ACC, p-P70S6K, P70S6K, α-tubulin for the determination of their cellular levels in HRV1B-infected and non-infected HeLa cells after treatment with budesonide (125 μM and 25 μM) for 8 h. (B) HRV1B-infected HeLa cells were treated with budesonide and the mitochondrial ROS were measured by MitoSOX. (C) Mitochondrial ROS were measured based on MitoSOX mean fluorescence. Results are shown as means ± SEM. ns; not significant, $^{**}P < 0.01$, $^{***}P < 0.001$ for comparisons with non-infected control (Ctrl), $^{++}p < 0.01$, $^{+++}p < 0.001$ for comparisons with HRV1B-infected vehicle (Veh).

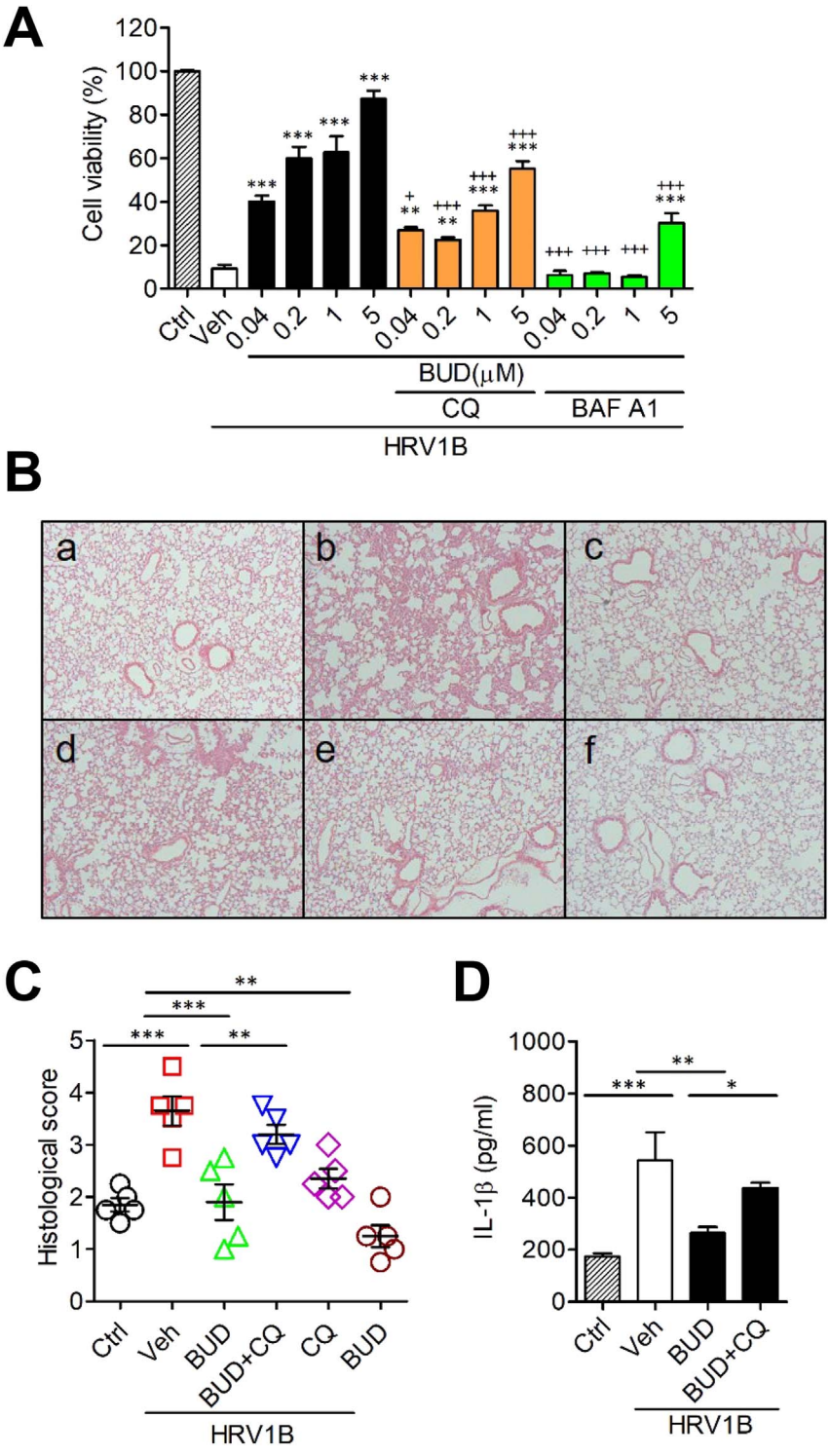

Fig. 6. Blockade of autophagy reduced antiviral activity of budesonide. (A) HeLa cells were infected with HRV1B at an MOI of 10 and treated with budesonide in the presence of 100 μM chloroquine or 0.025 μM bafilomycin A1. Cells were incubated for 48 h, and cell viability was measured by SRB assay. Results shown represent means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparisons with HRV1B infected vehicle, $^{+}P < 0.05$ and $^{+++}P < 0.001$ for comparisons between chloroquine and bafilomycin A1 treatment at the same dose of budesonide alone. (B–D) BALB/c mice were infected with HRV1B, and 0.1 mg/kg budesonide and/or 60 mg/kg chloroquine was intranasally administered. (B) Lung pathology was assessed after H&E staining in uninfected mice (a), HRV1B-infected mice (b), HRV1B-infected mice treated with budesonide alone (c), HRV1B-infected mice treated with budesonide and chloroquine (d), HRV1B-infected mice treated with chloroquine (e), uninfected mice treated with budesonide (f). (C) Pathological scores were determined according to the severity of edema, hemorrhage and immune cell infiltration. (D) The IL-1β levels were analyzed in lung tissue homogenates. Results are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Interestingly, the levels of IL-1β were decreased by budesonide treatment. The IL-1β level was determined at 8 h post-infection to assess early events after HRV infection as previously reported (Song et al., 2017; Shim et al., 2016). Partial blockade of IL-1β by Anakinra ameliorates the replication of HRV, whereas complete blockade of IL-1β in IL-1R knockout mice aggravated the infection, suggesting the critical need for precise regulation of IL-1β production in the management of HRV infection. We found that budesonide treatment induced antiviral activity against rhinovirus via GCR-dependent autophagy activation. Autophagy activation directly mediated the elimination of viruses, or led to increased degradation of regulatory proteins associated with NF-κB signaling or inflammasome formation.

Contrary to a previous report suggesting the inflammasome activation in bronchial cells by HRV infection (Triantafilou et al., 2013), we failed to observe any significant inflammasome activation based on the cleavage of caspase-1. Further, budesonide failed to reduce the levels of NALP3, while it decreased pro-IL-1β expression in HRV-infected macrophages. The discrepancy between pro-IL-1β and NALP3 expression following budesonide treatment suggested the possibility of additional regulatory pathways more than NF-κB pathways targeted by budesonide. We found that HRV1B infection slightly increased autophagy pathway, and budesonide treatment further increased the autophagy

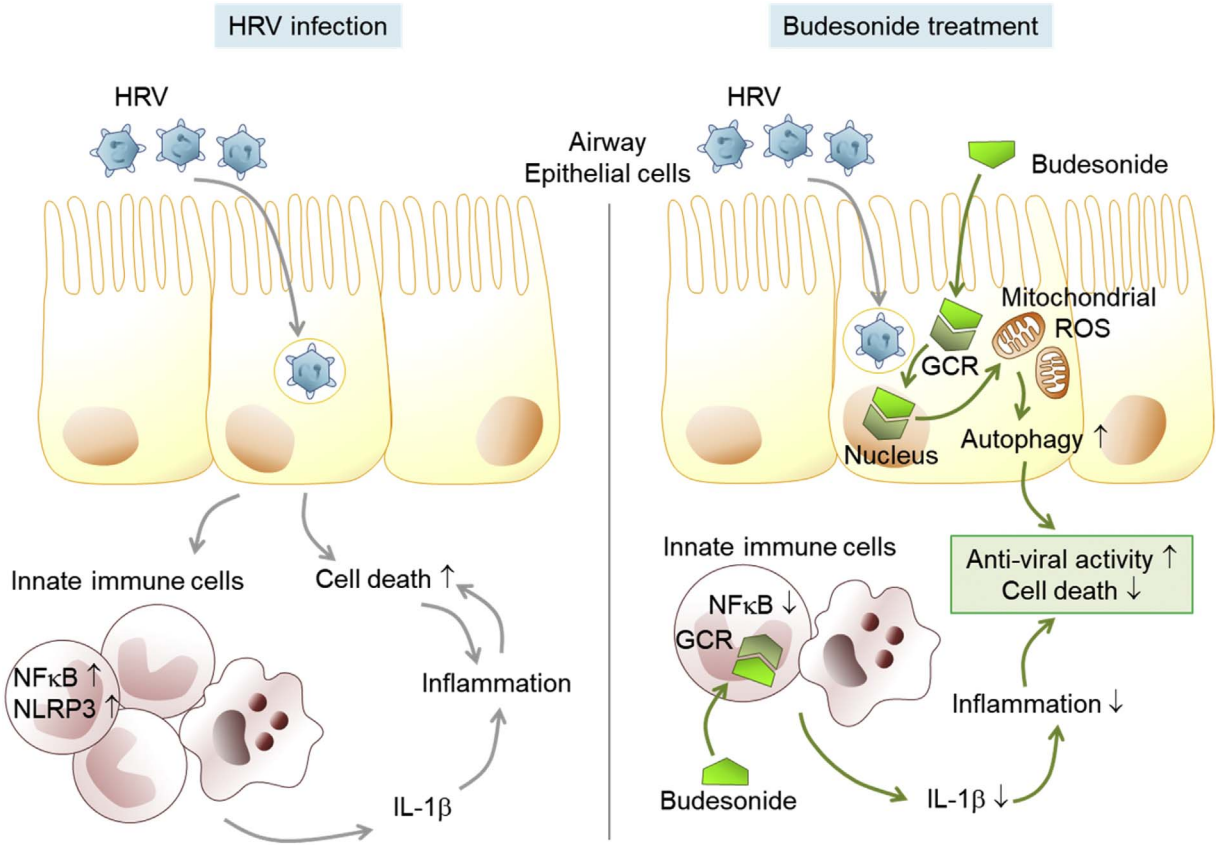

Fig. 7. A model of budesonide-mediated anti-viral activity. HRV infection increases inflammation and cell death, and results in the production of inflammatory cytokines such as IL-1β by innate immune cells. Upon budesonide treatment, HRV replication and proinflammatory cytokine production were decreased via mitochondrial ROS and autophagy-dependent pathways. At the same time, the binding of budesonide and GCR in innate immune cells decreased IL-1β production. Consequently, budesonide treatment reduces HRV replication both by reducing airway inflammation and increasing autophagic activation.

activation. We found that autophagy was AMPK- and mTOR pathway-independent, and apparently, the increased mitochondrial ROS levels may trigger autophagy activation by budesonide.

Autophagy is a cellular homeostatic mechanism that facilitates autophagosome formation to encapsulate the cellular components (Fader and Colombo, 2009). Autophagy plays a role in several aspects of cellular physiology and defends against various forms of cellular stress associated with inflammation (Levine et al., 2011). Autophagy can also inhibit the growth and survival of several viruses (Deretic and Levine, 2009), including vesicular stomatitis virus (Liu et al., 2005) and herpes simplex virus 1 (HSV-1) (Orvedahl et al., 2007). In previous reports, it was shown that HRV2 and HRV14 induce autophagy (Jackson et al., 2005; Klein and Jackson, 2011). Conversely, autophagy pathways are hijacked by a few viruses during replication. Interestingly, it was suggested that picornaviruses including rhinoviruses and coxsackie B virus utilize LC3-coated phosphatidylserine-rich membrane for their release (Munz, 2017). Thus, the role of autophagy in antiviral activity remains uncertain and needs additional investigation.

In the current study, however, we showed that the antiviral activity of budesonide was inhibited *in vitro* by chloroquine and bafilomycin A1 treatment, and administration of chloroquine also inhibited the HRV-associated lung pathology *in vivo*. Although the mechanism of autophagy activation by mitochondrial ROS is still unclear, it was recently reported that deoxypodophyllotoxin induced autophagy not through the PI3K/AKT/mTOR pathway, but by activating mitochondrial ROS-induced ERK/MAPK signaling (Kim et al., 2017). Further, dexamethasone enhanced ROS accumulation in osteoclast precursors and led to autophagy, but the precise mechanism was not provided (Shi et al., 2015).

Collectively, our results suggest that autophagy activation by budesonide inhibits both HRV replication and inflammation including IL-1β production through GCR-mediated pathway (Fig. 7), suggesting that budesonide represents a possible therapeutic agent against rhinovirus infection.

Conflict of Interest

The authors declare they have no conflicts of interest.

Acknowledgements

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