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PI3K-delta mediates double-stranded RNA-induced upregulation of B7-H1 in BEAS-2B airway epithelial cells

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ABSTRACT

Airway viral infection disturbs the health-related quality of life. B7-H1 (also known as PD-L1) is a coinhibitory molecule associated with the escape of viruses from the mucosal immunity, leading to persistent infection. Most respiratory viruses generate double-stranded (ds) RNA during replication. The stimulation of cultured airway epithelial cells with an analog of viral dsRNA, polyinosinic-polycytidylic acid (poly IC) upregulates the expression of B7-H1 via activation of the nuclear factor κB(NF-κB). The mechanism of upregulation was investigated in association with phosphatidylinositol 3-kinases (PI3Ks). Poly IC-induced upregulation of B7-H1 was profoundly suppressed by a pan-PI3K inhibitor and partially by an inhibitor or a small interfering (si)RNA for PI3Kδ in BEAS-2B cells. Similar results were observed in the respiratory syncytial virus-infected cells. The expression of p110δ was detected by Western blot and suppressed by pretreatment with PI3Kδ siRNA. The activation of PI3Kδ is typically induced by oxidative stress. The generation of reactive oxygen species was increased by poly IC. Poly IC-induced upregulation of B7-H1 was attenuated by *N*-acetyl-L-cysteine, an antioxidant, or by oxypurinol, an inhibitor of xanthine oxidase. Poly IC-induced activation of NF-κB was suppressed by a pan-PI3K inhibitor but not by a PI3Kδ inhibitor. These results suggest that PI3Kδ mediates dsRNA-induced upregulation of B7-H1 without affecting the activation of NF-κB.

1. Introduction

The common respiratory viruses are the rhinovirus and the respiratory syncytial virus (RSV) [1,2]. They have genomic single-stranded RNA and generate double-stranded (ds) RNA in their host cells. dsRNA triggers innate immune responses via its binding to recognition receptors, including TLR3, the retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) [3–5]. TLR3 recognizes viral dsRNA and a synthetic dsRNA, polyinocinic polycytidilic acid (poly IC), in the endosome. Both RIG-I and MDA5 recognize dsRNA in the cytoplasm. RIG-I recognizes 5′ triphosphate motif of RSV single-stranded RNA as well as its dsRNA [6], while MDA5 recognizes rhinovirus dsRNA and poly IC. Additionally, rhinovirus is recognized by RIG-I and TLR3 [7]. Subsequent signaling leads to the production of type I IFNs, proinflammatory cytokines, and the upregulation of costimulatory and coinhibitory molecules.

Adapted antiviral immunity is regulated by the interactions between antigen-presenting cells and T cells. The interactions are characterized by the binding of an array of costimulatory and coinhibitory molecules, including B7-family molecules, and their putative receptors [8,9]. B7-H1 belongs to the B7 family and shares its receptor, programmed death-1 (PD-1), with B7-DC. B7-H1 and B7-DC are also known as PD-1 ligand 1 (PD-L1) and 2 (PD-L2), respectively. PD-1 is inducibly expressed in T cells and B cells during their activation, and its ligation to B7-H1 suppresses the activated status. In a mouse model of chronic viral infection and circulating dendritic cells in patients with chronic hepatitis B, blockade of the B7-H1/PD-1 pathway restored the function of cytotoxic T lymphocytes (CTLs) and decreased the viral load [10,11]. Chronic RSV infection was detected in patients with chronic airway disease and associated with the accelerated decline of lung function [12]. Human airway epithelial cells expresse a low level of B7-H1 and poly IC stimulation upregulated B7-H1 expression [13]. The blockade of B7-H1 on RSV-infected epithelial cells in co-culture with $CD8^+$ T cells enhanced antiviral CTL activity [14]. Furthermore, treatment of mice with anti-B7-H1 mAb prevented virus-specific CTL impairment and reduced viral titers during infection with human metapneumovirus [15]. dsRNA-induced upregulation of B7-H1 may prevent the infected epithelial cells from the attack by CTLs, thereby facilitating the spread of a virus around the neighboring cells.

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Viral infections activate a variety of kinases in the host cells. Their representatives may be mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinases (PI3Ks). They not only mediate proliferation and survival of the cells but also contribute to the induction of immune responses. We herein demonstrate that PI3Kδ mediates dsRNA-induced upregulation of B7-H1 in BEAS-2B airway epithelial cells.

2. Materials and methods

2.1. Reagents

SB203580 as the selective inhibitor of p38MAPK, SP600125 as the selective inhibitor of c-Jun *N*-terminal kinase (JNK), U0126 as the selective inhibitor of MAPK/extracellular signal-regulated kinase (ERK) kinase 1 and MAPK/ERK kinase 2, wortmannin as the pan inhibitor of class I PI3K, rotenone, oxypurinol and poly IC were purchased from SIGMA–ALDRICH (St. Louis, MO, USA). IC87114 as the selective inhibitor of PI3Kδ was purchased from BioVision (Mountain View, CA, USA). AS604850 as the selective inhibitor of PI3Kγ and *N*-acetyl-L-cysteine (NAC) were purchased from Wako (Osaka, Japan). Lipofectamine® 2000 and Alexa fluoro488® goat anti-mouse IgG Ab were purchased from Invitrogen (San Francisco, CA, USA). Rabbit anti-human phosphorylated IκBα Ab was purchased from Cell Signaling (Beverly, MA, USA). Mouse anti-human NF-κB p65 mAb, and mouse anti-human β-actin mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated mouse anti-human B7-H1, anti-human B7-DC and anti-mouse B7-H1 mAbs were purchased from eBioscience (San Diego, CA, USA). Mouse anti-p110δ mAb and streptavidin–phycoerythrin (SAv–PE) conjugate were purchased from BD Biosciences (San Jose, CA, USA). p110δ PI3Kδ siRNA and negative control siRNA were purchased from Ambion (Carlsbad, CA, USA). Gene Silencer® was purchased from Genlantis (San Diego, CA, USA).

2.2. Epithelial cell line

The BEAS-2B cells were cultured by using DMEM/F12. The medium contained 10% FBS, penicillin (100 U/ml), and streptomycin (100 ng/ml).

2.3. Preparation of bone marrow-derived dendritic cells

Experimental procedures were approved by the animal research ethics committee of Kyushu University. Bone marrow-derived dendritic cells (BMDCs) were prepared from BALB/c mice with recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN, USA) at 10 ng/ml for 4–6 days after the initiation of culture. On day 7, BMDCs were stimulated with poly IC.

2.4. Stimulation of BEAS-2B cells with dsRNA or RSV

Semi-confluent cells were treated with drugs or siRNA and stimulated by administration of poly IC (3 μg/ml) in the culture medium. Otherwise, cells were transfected with poly IC (10 ng/well) by using Lipofectamin® 2000 in Opti MEM®I medium (Invitrogen, San Fransisco, CA, USA), or infected with RSV at a multiplicity of infection (MOI) of 1. B7-H1 expression was assessed 24 h after the stimulation.

2.5. Flow cytometry

Cells were incubated with biotynilated anti-B7-H1 mAb, followed by staining with SAv–PE conjugate and then processed for flow cytometric analysis. Flow cytometric analysis was performed using a FACSCalibur flow cytometer equipped with CELLQuest software (BD Biosciences). The mean fluorescence intensity (MFI) was compared with control staining using an irrelevant isotype-matched Ab.

2.6. Western blot analysis

Whole-cell protein lysates were prepared by solubilizing in a lysis buffer (20 mM Tris–HCl, pH 7.8, 138 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1% Nonidet P-40, and 1% Triton X-100) with a protease inhibitor cocktail. The lysates were denatured, separated by 12% SDS–polyacrylamide gels, and transferred to Immunobilon P membranes. Membranes were blocked in 5% skimmed milk in TBS-Tween (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0) for 1 h. Blotting was performed with an antibody for phosphorylated-IκBα, p110δ, or β-actin at 4 °C overnight. Membranes were washed and then incubated with the secondary, peroxidase-coupled antibody for 30 min. Membranes were washed, and specific bands were visualized using a SuperSignal® West Femto Trial kit (Thermo, Rockford, IL, USA).

2.7. Immunofluorescence analysis

BEAS-2B cells were divided into a 6-well plate containing poly-lysine-coated 22 × 22 mm coverslips and stimulated with poly IC for 1 h. Coverslips were washed in PBS, fixed with 4% paraformaldehyde, and stained for NF-κB p65. Anti-NF-κB p65 mAb was diluted with 10% BlockAce® (DSfarmabiomedical, Osaka, Japan), added to cells, and incubated overnight at 4 °C. Cells were washed in 0.1% Tween20/PBS and coincubated with Alexa fluor488®–conjugated secondary antibody for 4 h in the dark at 4 °C. Cells were washed, and coverslips were mounted onto slides using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA); localization of the NF-κB p65 protein was detected by direct fluorescence microscopy (KEYENCE, Osaka, Japan).

2.8. Measurement of reactive oxygen species (ROS)

BEAS-2B cells ware cultured in 96-well plates. After washing by Hanks' balanced salt solution, cells were loaded with 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluoroscein diacetate (CMH_2-DCFDA). After 30 min, they were replaced with a fresh medium and stimulated with poly IC. Intracellular ROS were measured using a fluorescent plate reader (Wallac 1420 ARVO). Increases in fluorescence at 5 min ware calculated, and background fluorescence of the cell-free medium was subtracted.

2.9. Measurement of mitochondrial ROS

Mitochondria-associated ROS levels were measured for cells stained with MitoSOX (Molecular Probes/Invitrogen, Eugene, OR, USA) at 5 μM for 15 min at 37 °C. Cells were washed with PBS, trypsinized, and resuspended in PBS for flow cytometry.

2.10. Data analysis

Values were expressed as the means ± SEM. Differences were analyzed using an ANOVA with Bonferroni's correction. *P*-values < 0.05 were accepted as significant.

3. Results

3.1. Poly IC- and RSV-induced upregulation of B7-H1 is mediated by PI3K

Unstimulated BEAS-2B cells express a low level of B7-H1 [13,16]. The administration of poly IC to the culture medium

significantly upregulated the expression of B7-H1. The upregulation was abolished by pretreatment of cells with chloroquine or TLR3 siRNA, indicating the involvement of TLR3-recognized endosomal pathways [16]. The upregulation of B7-H1 was profoundly suppressed by wortmannin (Fig. 1A). Viral dsRNA is alternatively sensed via RIG-I/Mda5-recognized cytoplasmic pathways. To mimic those responses, cells were transfected with poly IC by using lipofectamine. The treatment with lipofectamine alone did not affect the baseline expression of B7-H1 (data not shown). The expression of B7-H1 was upregulated by poly IC transfection, which was significantly suppressed by wortmannin (Fig. 1B). To evaluate biological relevance, the effect of wortmannin was examined for cells infected with RSV (Fig. 1C). The expression of B7-H1 was significantly upregulated by infection with RSV but not with UV-irradiated RSV. The upregulation of B7-H1 was abolished by wortmannin. Previous investigations showed that PI3K negatively regulates the production of IL-12 and IFN-β in dendritic cells stimulated with poly IC [17]. The effect of wortmannin was examined for BMDCs (Fig. 1D). The expression of B7-H1 was unaffected by poly IC at 3 μg/ml. The expression of B7-H1 was significantly upregulated by wortmannin regardless of poly IC stimulation although the magnitude of upregulation was higher in poly IC-stimulated BMDCs than in unstimulated BMDCs. Hence, PI3K has opposing role in the regulation of B7-H1 expression between epithelial cells and dendritic cells. The opposing role was also shown in the expression of B7-DC (Fig. 1E–G). Next, the effects of MAPK inhibitors were examined. The upregulation of B7-H1 by the administration of poly IC to the culture medium was only partially suppressed by pretreatment with U0126 but not with SB203580 or SP600125 (Fig. 1H).

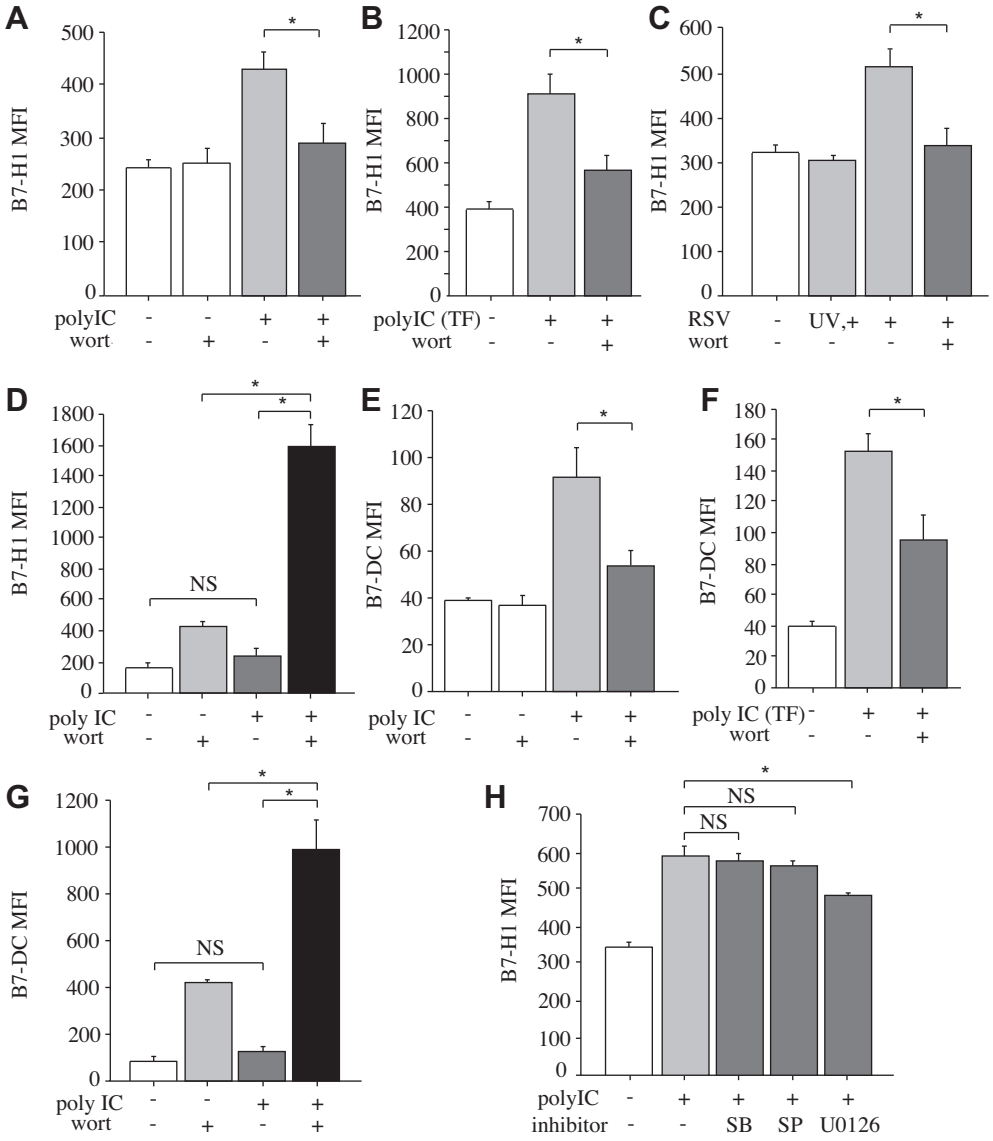

Fig. 1. Effects of inhibitors on poly IC- or RSV-induced upregulation of B7-H1. (A), (E) and (H) BEAS-2B cells were pretreated with wortmannin (wort) (1 μM), SB203580 (SB) (10 μM), SP600125 (SP) (10 μM), U0126 (10 μM), or vehicle alone for 0.5 h and administered with poly IC (3 μg/ml) to the culture medium for 24 h, and then processed for B7-H1 or B7-DC analysis. (B) and (F) BEAS-2B cells were pretreated with wortmannin or vehicle alone for 0.5 h and transfected with poly IC (10 ng/well) using Lipofectamine® 2000. After 24 h of transfection (TF), cells were processed for B7-H1 or B7-DC analysis. (C) BEAS-2B cells were pretreated with wortmannin or vehicle alone for 0.5 h and infected with RSV (1 MOI) or UV-irradiated RSV. After 24 h of infection, cells were processed for B7-H1 analysis. (D) and (G) Murine bone marrow-derived dendritic cells were pretreated with wormannin or vehicle alone for 0.5 h and administered with poly IC (3 μg/ml) to the culture medium for 24 h, and then processed for B7-H1 or B7-DC analysis. Data are expressed as the means ± SEM of 4–5 experiments. $^{*}p < 0.05$. NS means statistically not significant.

3.2. Poly IC- and RSV-induced upregulation of B7-H1 is mediated by PI3Kδ

Class I PI3Ks are heterodimers comprising four catalytic isoforms (p110α, p110β, p110δ, or p110γ)[18]. PI3Kα and PI3Kβ are ubiquitously expressed and have crucial roles in cell kinesis and maintaining cell structure whilst previous studies showed that potentials of PI3Kδ and PI3Kγ may be limited to mediate inflammatory responses. The upregulation of B7-H1 by administration of poly IC to the culture medium, transfection of poly IC, or infection of RSV was unaffected by pretreatment with AS604850, a selective inhibitor of PI3Kγ (Fig. 2A–C). The upregulation of B7-H1 by administration of poly IC to the culture medium or infection of RSV, but not by transfection of poly IC, was significantly suppressed by pretreatment with IC87114, a selective inhibitor of PI3Kδ (Fig. 2D–F). Western blot study showed the expression of p110δ in whole-cell protein lysates of unstimulated cells (Fig. 2G). The expression of p110δ was suppressed by pretreatment of cells with p110δ siRNA but not by control siRNA. The poly IC-induced upregulation of B7-H1 was partially suppressed by pretreatment with p110δ siRNA (Fig. 2H).

3.3. Poly IC-induced upregulation of B7-H1 is mediated by xanthine oxidase-derived ROS

The activation of PI3Kδ is typically induced by oxidative stress [19,20]. The generation of intracellular ROS was increased by administration of poly IC to the culture medium (Fig. 3A). The poly IC-induced upregulation of B7-H1 was partially suppressed by pretreatment with NAC (Fig. 3B). Intracellular ROS are generated via the NADPH oxidase (NOX) system, the mitochondrial oxidative phosphorylation system, and the xanthine dehydrogenase/xan-

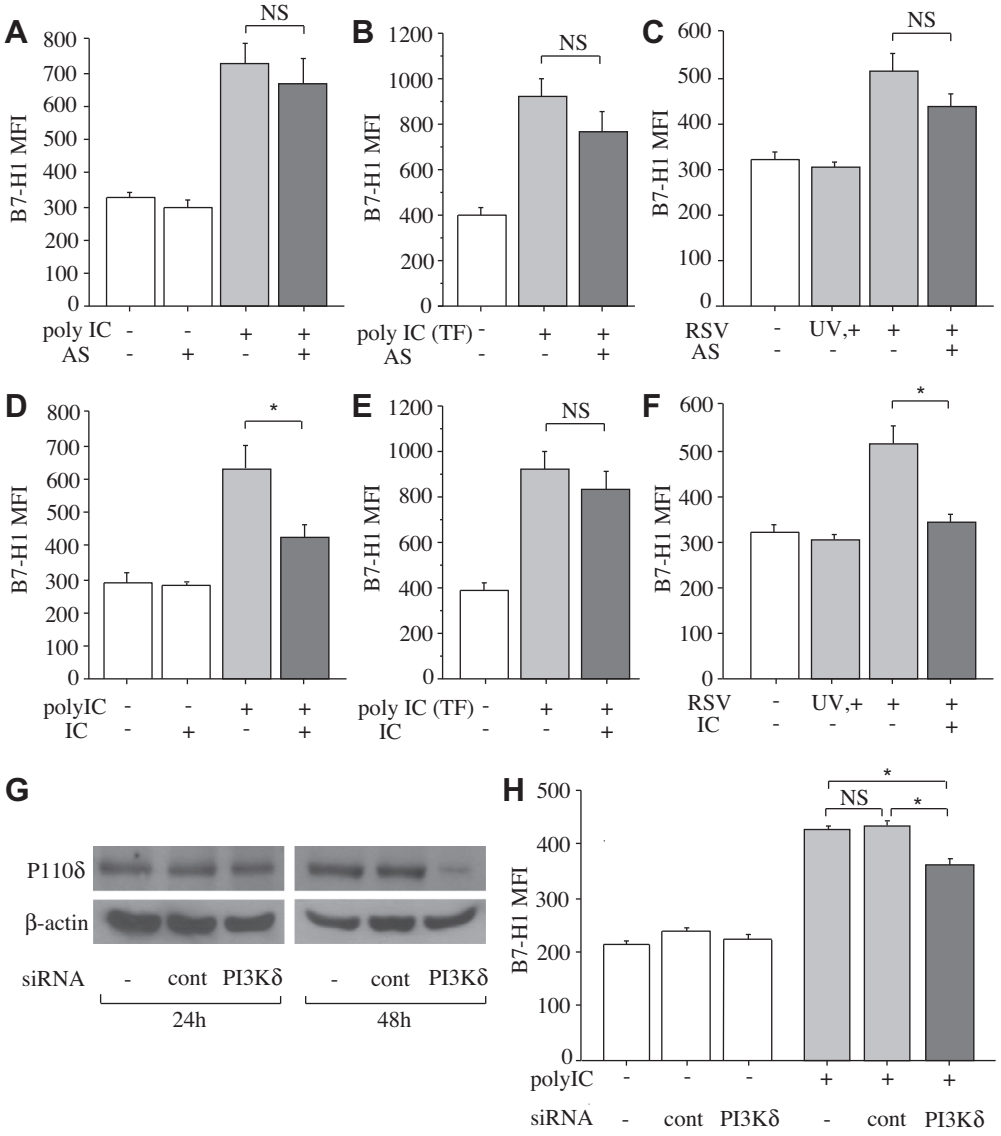

Fig. 2. Effects of inhibition for PI3Kγ or PI3Kδ on poly IC- or RSV-induced upregulation of B7-H1. (A) and (D) BEAS-2B cells were pretreated with AS604850 (AS)(10 μM), IC87114 (IC) (10 μM), or vehicle alone for 0.5 h and administered with poly IC to the culture medium for 24 h, and then processed for B7-H1 analysis. (B) and (E) Cells were pretreated with AS604850, IC87114, or vehicle alone for 0.5 h and transfected with poly IC. After 24 h of transfection, cells were processed for B7-H1 analysis. (C) and (F) Cells were pretreated with AS604850, IC87114, or vehicle alone for 0.5 h and infected with RSV or UV-irradiated RSV. After 24 h of infection, cells were processed for B7-H1 analysis. (G) Cells were transfected with p110δsiRNA (PI3Kδ) (75 nM/well), control negative siRNA (cont) for 4 h. After 24 or 48 h of RNAi, cellular extracts were processed for Western blotting. Data represents two independent experiments. (H) Cells were transfected with p110δsiRNA, control negative siRNA, or vehicle alone for 48 h, and processed for B7-H1 analysis. Data are expressed as the means ± SEM of 4–5 experiments. $^{*}p < 0.05$. NS means statistically not significant.

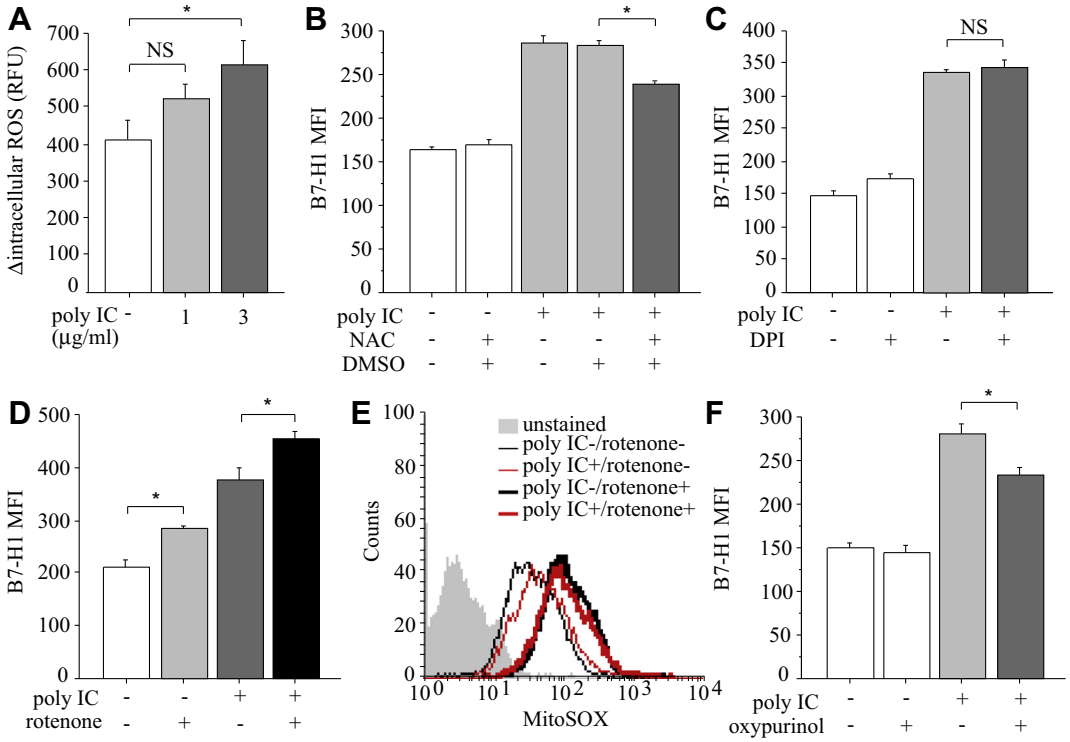

Fig. 3. Roles of ROS in poly IC-induced upregulation of B7-H1. (A) BEAS-2B cells were loaded with CMH_2-DCFDA and administered with poly IC to the culture medium for 5 min. Intracellular ROS were measured. (B), (C), (D), and (F) Cells were pretreated with *N*-acetyl-L-cysteine (NAC) (1 mM) for 2 h, diphenyleneiodonium chloride (DPI) (10 μM) for 0.5 h, rotenone (10 μM) for 0.5 h, oxypurinol (20 μM) for 4 h, or vehicle alone and administered with poly IC to the culture medium for 24 h, and then processed for B7-H1 analysis. Data are expressed as the means ± SEM of 6–9 experiments. $^{*}p < 0.05$. NS means statistically not significant. (E) Cells were pretreated with or without rotenone for 0.5 h and administered with or without poly IC to the culture medium for 24 h. Mitochondria-associated ROS levels were measured for cells stained with MitoSOX®. Data represents three independent experiments.

thine oxidase (XD/XO) system [21–23]. The poly IC-induced upregulation of B7-H1 was unaffected by pretreatment with diphenyleneiodonium chloride (DPI), a NOX inhibitor (Fig. 3C). Several studies reported that ROS were generated via the activation of dual NADPH oxidases/peroxidases 2 (Duox 2), a homolog of NOX, in poly IC-stimulated or rhinovirus-infected human airway epithelial cells [24]. However, no increase in mRNA or protein expression of Duox 2 was detected in BEAS-2B cells after poly IC stimulation in real-time PCR or Western blot analysis, respectively (data not shown). Rotenone is a mitochondrial complex I inhibitor and increases mitochondrial superoxide generation [25]. The expression of B7-H1 was upregulated by pretreatment of cells with rotenone regardless of poly IC stimulation (Fig. 3D). Although the mitochondrial superoxide generation was increased by rotenone, the generation was unaffected by poly IC (Fig. 3E). These results suggest that mitochondrial superoxide was not responsible for B7-H1 upregulation. The poly IC-induced upregulation of B7-H1 was significantly suppressed by pretreatment with oxypurinol, an inhibitor of XO (Fig. 3F).

3.4. PI3Kδ mediates poly IC-induced B7-H1 upregulation without affecting NF-κB activation

Given that activation of NF-κB is essential for poly IC-induced upregulation of B7-H1 [16], the effect of wortmannin or IC87114 on NF-κB activation was examined. The activation of NF-κB is initiated by phosphorylation of IκBα. Phosphorylated IκBα loses its binding activity to NF-κB, which allows free NF-κB translocate to the nucleus. In Western blot study, the amount of phosphorylated IκBα was decreased by wortmannin, but not with IC87114, AS604850, or U0126 (Fig. 4A and B). Immunofluorescence analysis demonstrated that the poly IC-induced nuclear translocation of NF-κB was prevented by wortmannin, but not by IC87114 (Fig. 4C). These results suggest that PI3Kδ mediates poly IC-induced B7-H1 upregulation without affecting NF-κB activation.

4. Discussion

PI3Ks are central players in many cellular responses, including movement of organelle membranes, alteration of cell morphology, and proliferation. Indeed, a similar association between B7-H1 and PI3K has been reported in several neoplasms [26,27]. PI3K is associated with incorporation of target particles into the cells by endocytosis [28]. We previously confirmed no expression of TLR3 protein on the cell surface of BEAS-2B cells while the expression is localized in endosome [29]. Lipofection for poly IC transfection also requires the step of endocytosis. An arising concern is that the suppression of B7-H1 upregulation by wortmannin might be due to failure of initial poly IC incorporation. This concern is less likely because it could not explain the contrasting enhancement of poly IC-induced B7-H1 upregulation by wortmannin in BMDCs.

The poly IC-induced upregulation of B7-H1 was partially suppressed by IC87114, a PI3Kδ inhibitor. This inhibition was unexpected because the reports of p110δ expression had been largely restricted to hematopoietic cells [30]. Western blot analysis, however, detected substantial expression of p110δ in BEAS-2B cells, which was suppressed by p110δ siRNA. Furthermore, poly IC-induced upregulation was suppressed by p110δ siRNA. To our knowledge, this is the first report to demonstrate the biologically relevant existence and function of PI3Kδ in the lineage of airway epithelial cell. IC87114 suppressed the upregulation of B7-H1 induced by administration of poly IC to the culture medium, but not by its transfection, suggesting that PI3Kδ works in TLR3-recog-

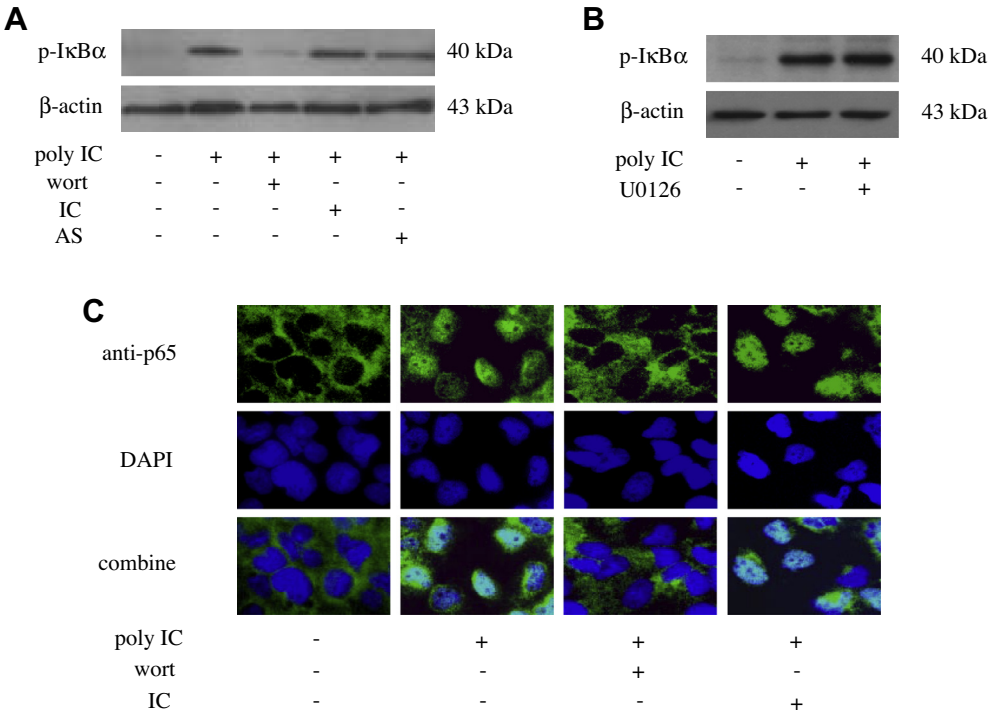

Fig. 4. Effects of chemical inhibitors for on the activation of NF-κB. (A) and (B) BEAS-2B cells were pretreated with wortmannin (1 μM), IC87114 (10 μM), AS604850 (10 μM), U0126 (10 μM), or vehicle alone for 0.5 h and administered with poly IC to the culture medium for 60 min. Cellular extracts were processed for Western blotting. Data represents two independent experiments. (C) Cells were pretreated with wortmannin, IC87114, or vehicle alone for 0.5 h and administered with poly IC to the culture medium for 60 min. Cells were stained with anti-NF-κB p65 mAb and Alexa fluoro488®-conjugated Ab. The nucleus was counterstained with DAPI. NF-κB p65 protein was detected by direct fluorescence microscopy.

nized pathway, but not in RIG-I/Mda5-recognized pathway. Considering clinical implication, it is noteworthy that the upregulation of B7-H1 by RSV infection was markedly suppressed by IC87114. Immune responses to RSV infection take an initial step recognized by RIG-I and a following step by TLR3 [31]. IC87114 may suppress B7-H1 upregulation by acting on PI3Kδ-dependent component in the latter step.

Contrasting to BEAS-2B cells, poly IC-induced upregulation of B7-H1 was augmented by wortmannin in BMDCs. In a majority of studies using dendritic cells and macrophages, PI3K inhibitors augment TLR-ligands-induced activation of NF-κB [18]. B7-H1 protects cancer cells or virus-infected tissue cells from the attack by CTLs while B7-H1 on dendritic cells has inhibitory or stimulatory effect on T cells, depending on immune situations. These cell-specific tasks of B7-H1 might be related to the different mode of actions in PI3K signaling.

The pharmaceutical development of PI3K inhibitors has been accelerated for targeting neoplastic diseases and inflammatory diseases. Considering that ubiquitous distribution of PI3Kα and PI3Kβ and their crucial roles in the homeostasis, the application of pan-PI3K inhibitors for clinical use might be threatened by serious adverse effects. With this regard, the limited distribution and function of PI3Kδ may be a promising pharmaceutical target for preventing the persistent viral infection.

Acknowledgments

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