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Inhibition of Akt kinase activity suppresses entry and replication of influenza virus

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ABSTRACT

The possibility of the pandemic spread of influenza viruses highlights the need for an effective cure for this life-threatening disease. Influenza A virus, belonging to a family of orthomyxoviruses, is a negative-strand RNA virus which encodes 11 viral proteins. A numbers of intracellular signaling pathways in the host cells interact with influenza the viral proteins, which affect various stages of viral infection and replication.

In this study, we investigated how inhibition of Akt kinase activity impacts on influenza virus infection by using "*Akt-in*", a peptide Akt inhibitor. In PR8 influenza-infected A549 cells, Akt interacted with the NS1 (Non structural protein 1), and hence increased phosphorylation of Akt kinase activity and NS1. Treatment of cells with either "TCL1- or TCL1b-based *Akt-in*" efficiently suppressed Akt kinase activity while decreasing the levels of phosphorylated NS1; this, in turn, inhibited viral replication in a dose- and time-dependent manner. The inhibitory effect on viral replication appears to not be due to inhibition of the production of inflammatory cytokines, including IL-6 and IL-8, in the host cells. Inhibition of Akt kinase activity in the host cells inhibited the efficiency of viral entry, which is associated with decreased levels of phosphorylated glycogen synthase kinase 3, a substrate of Akt. Thus inhibition of Akt kinase activity in host cells may have therapeutic advantages for influenza virus infection by inhibiting viral entry and replication.

1. Introduction

The serine/threonine kinase Akt, which belongs to the cAMP dependent, cGMP dependent, and protein kinase C (AGC) group, was originally identified from the Akt8 acute transforming retrovirus, which causes mouse thymoma [1,2].

In response to various extracellular stimuli, Akt is activated by the lipid products of phosphoinositide 3′-kinase (PI3K), namely PtdIns (3,4,5) P_3 and its immediate breakdown product PtdIns (3,4) P_2; PI3K phosphorylates the 3′-OH position of the inositol core of inositol phospholipids [3,4]. The binding of PtdIns (3,4,5) P_3 to the Akt-pleckstrin homology (Akt-PH) domain within variable loop 1 (the loop between the β1 and β2 strands) and its subsequent translocation to the plasma membrane, allows phosphoinositide-dependent kinase-1 (PDK1) to access and trigger phosphorylation at a threonine residue (Thr308) within the catalytic domain of Akt [1,2,5]. The mTOR-Rictor complex 2 (TORC2) has also recently been shown to be involved in a major activation mechanism underlying the phosphorylation of Ser473 in the carboxy-terminal hydrophobic motif [6].

Once activated, Akt controls downstream cellular responses, including the regulation of activities related to anti-apoptosis, proliferation, cell cycle, glycogen synthesis, angiogenesis, and telomerase. Thus, PI3K–Akt pathways underlie the pathogenesis of various human diseases. It is noteworthy that the PI3K–Akt pathway underlies clinical manifestation of different stages of infection by virus such as Epstein–Barr, hepatitis C, hepatitis B, and human immunodeficiency virus (HIV), as well as influenza virus [2,7,8].

In recent years, pandemic influenza caused by H1N1 avian influenza viruses have emerged, and now appear to have spread across many regions throughout the world [9]. The influenza virus consists of eight segmented minus-stranded RNAs which encode 11 known proteins. A number of intracellular signaling pathways in the host cells has been shown to interact with viral proteins, a process that is critical for viral replication [9]. Among the 11 influenza proteins, non-structural protein 1 (NS1, encoded on segment 8), a 26-kDa protein, has been implicated in the regulation of several biological functions, including regulation of apoptosis suppression in the host immune responses, through NF-κB-dependent mechanisms [10–12].

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Recently, we have demonstrated physical and functional interaction of influenza NS1 protein with Akt, a core intracellular survival regulator and the major effector molecules of PI3K [7]. The RNA-binding domain of NS1 interacts with Akt-PH domain; this consequently enhances Akt kinase activity and results in phosphorylation of NS1 at Thr215. The Akt-NS1 functional interaction possibly facilitates the symbiotic relationship between the host cells and the virus, promoting cell survival for influenza viral replication [7].

In addition to Akt, influenza NS1 is also known to interact with the p85β regulatory subunit of PI3K by binding directly to the SH3 and C-terminal SH2 domain of p85; this consequently activates the PI3K–Akt pathway [13]. Therefore, influenza A virus NS1 protein activates the PI3K–Akt pathway in multiple steps during viral infection, so modulating the cellular responses of host cells.

Various human host cell factors are known to be required for influenza replication [14,15]. Intracellular PI3K–Akt pathways not only regulate very early steps during viral entry, but also suppress premature apoptosis at later stages of infection [8,9,13]. PI3K–Akt pathways play a critical role in uptake during viral entry, prevention of premature apoptosis, and viral RNA expression and RNP localization [13,16,17]. Virally infected cells can presumably avoid their own virus-induced death by effectively activating the PI3K–Akt pathway, which may allow scavengers to control the host cell-to-cell infectious process. Therefore, inhibitors of this cellular signaling pathway could represent a new promising antiviral therapy against influenza infection [8,15,18,19].

In this report, we pursued this proposed therapeutic intervention by using Akt inhibitors "*Akt-in*" [20–22] to investigate and clarify whether and how suppression of the PI3K–Akt pathway in host cells has a beneficial impact on controlling the influenza infection, with a view to therapeutic benefit against influenza virus infection.

2. Materials and methods

2.1. Cell lines, viruses, antibodies and pharmacological inhibitors

A549 and MDCK cells (ATCC) were cultured in RPMI-1640 (Sigma) or DMEM (Sigma) supplemented with 10% FBS. Influenza A/Puerto-rico/8/34 (H1N1, PR8) virus strain was provided by Dr. Kida. LY294002 (Sigma), TAT-FLAG (YGRKKRRQRRR-DYKDDDDK), TAT-TCL1-*Akt-in* (YGRKKRRQRRR-AVTDHPDRLWAWEKF) [20], and TAT-TCL1b-*Akt-in* (YGRKKRRQRRR-RLGVPPGRLWIQRPG) [21] were obtained from (MBL), Anti-Akt, anti-phospho-Ser473 Akt, anti-phospho-Ser308Akt, anti-phospho-Akt substrate, anti-phospho-GSK3α/β, anti-GSK3α/β antibodies (Cell Signaling Technology), anti-α-tubulin antibody (Sigma), anti-Influenza A NS1 antibody (Santa Cruz), and nucleoprotein antibody (BEI resources) were used in this study.

2.2. Influenza virus infection and plaque assays

A549 cells (ATCC) were infected with PR8 virus (MOI = 0.1) for 1 h. The viral supernatant was replaced with RPMI-1640, BSA, and trypsin. For the plaque assay, MDCK cells were incubated with the viral culture supernatant for 1 h, overlaid with agarose, and the numbers of plaques were counted.

2.3. Co-immunoprecipitation experiment of PR8-infected cells

A549 cells were infected with PR8 (MOI = 1) for 18 h, harvested, lysed, and immunoprecipitated with the indicated antibodies and immunoblotted using anti-NS1 antibody [7].

2.4. Immunofluorescent staining

A549 cells were incubated with the indicated inhibitors for 30 min before PR8 infection (MOI = 0.1), fixed, stained with the indicated antibodies or DAPI, and examined using a confocal microscopy (Fluoview FV-1000, Olympus).

2.5. Measurement of cytokines

The protein levels of IL-6 and IL-8 in the supernatant of PR8-infected A549 cells were measured using ELISA MAX Deluxe Sets (BioLegend).

2.6. Statistical analysis

Results were statistical analyzed using paired *t*-tests.

3. Results

3.1. Influenza virus infection enhances Akt kinase activity in PR8-infected A549 cells

The PI3K–Akt network has been shown to underlie clinical manifestations of various stages of viral infection [2,7,13]. Previously, we have shown that NS1 of influenza A virus interacts directly with Akt and enhances Akt kinase activity [7].

Influenza virus NS1 has been shown to antagonize the cellular immune response and is implicated in virulence by activating PI3K–Akt signaling pathway as one of the major downstream effectors of host cells that enhance efficient replication [10,12,13]. These observations prompted us to examine how infections of cells with PR8 influenza virus can alter the levels of Akt phosphorylation and that of its substrate, NS1 [7]. To this end, A549 cells were infected with PR8 and the levels of Akt and NS1 phosphorylation were examined. Indeed, we found that PR8 infection of A549 cells significantly induced levels of Akt phosphorylation, which was associated with increased phosphorylation of NS1 (Fig. 1A).

NS1 is also known to activate PI3K through its interaction with p85β, a regulatory subunit of PI3K, resulting in the phosphorylation of Akt [11,23]. Highly conserved tyrosine motifs of NS1 play a role in the interaction of this protein with p85β and in the activation of PI3K [24]. Given the observation that NS1 interacts directly with Akt and enhances Akt kinase activity [7], the enhanced phosphorylation of Akt and NS1 (Fig. 1A) is possibly due to the direct interaction of endogenous Akt with NS1 in PR8-infected A549 cells, as demonstrate by co-immunoprecipitation assays (Fig. 1B).

3.2. Inhibition of Akt kinase activity by TCL1- or TCL1b-based peptide inhibitors efficiently suppresses viral replication

Modulation of human host factors is important for regulating influenza virus replication [14]. PI3K–Akt signaling is known to play a role in various stages of influenza virus replication [13,16,17]. Since Akt signaling involves many aspects of cellular responses – such as those involving anti-apoptosis, proliferation, protein translation, metabolism and cell cycle – PI3K–Akt pathways provide a promising therapeutic target for human viral infection [11,15]. Given the fact that PR8 infection induces the activation of Akt and its viral protein substrate NS1, we next asked how activation of Akt and NS1 may affect the viral replication upon influenza infection.

Based on the structural–functional analysis of the TCL1-Akt protein complex, we previously identified a TCL1 and TCL1b-structure based-peptide, named TCL1-*Akt-in* (a TCL1-based Akt inhibitor

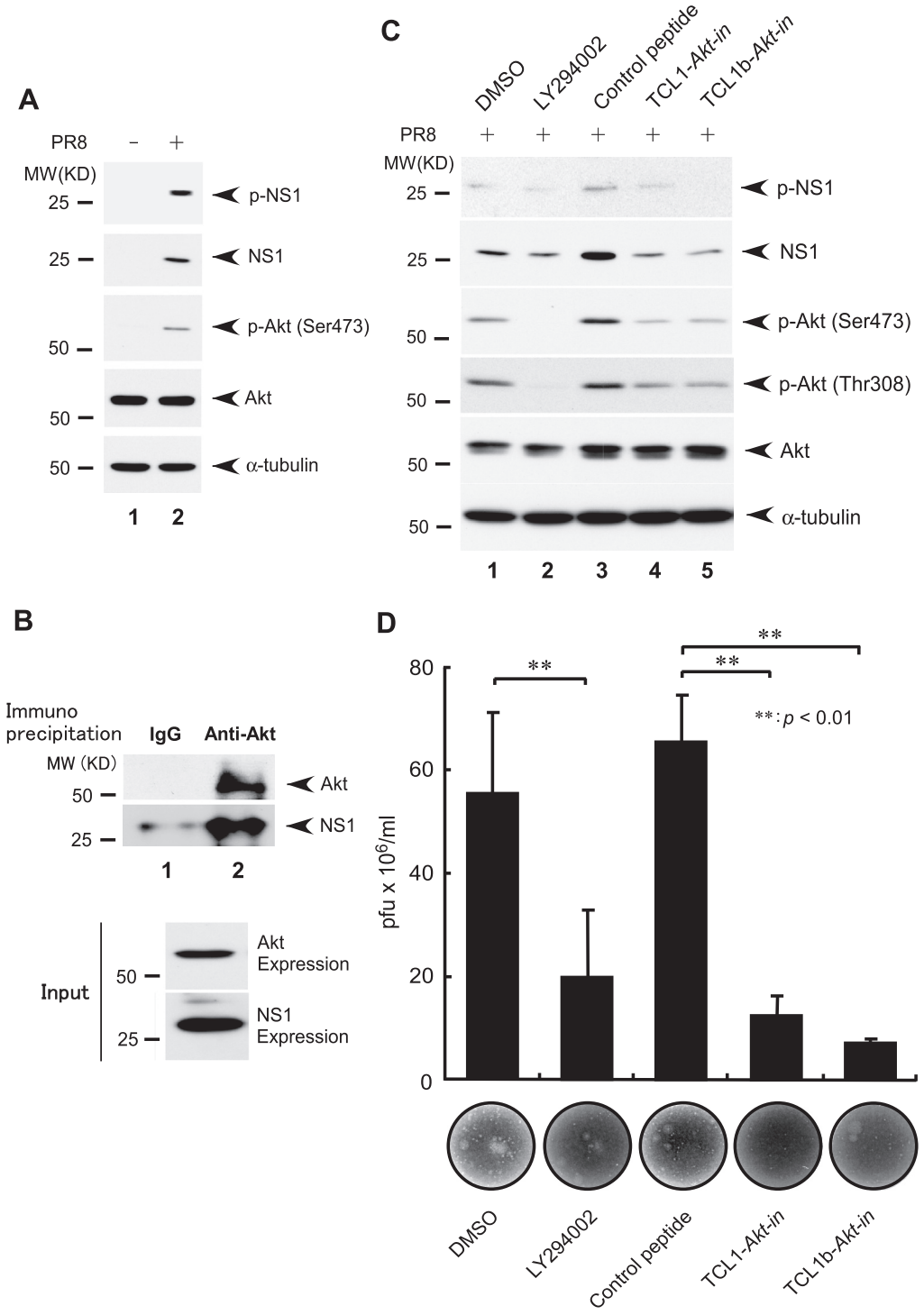

Fig. 1. (A) Infection A549 cells with PR8 increased Akt kinase activity, which is associated with increased levels of NS1 phosphorylation. (B) Endogenous Akt co-immunoprecipitated with influenza NS1 protein (lane4, second panel from the top) in PR8-infected A549 cells. Note that no NS1 protein could be immunoprecipitated by the control antibody (lane1, top panels). Expression of the NS1 protein and endogenous Akt of the PR8-infected cells are shown in the lower panels as internal controls. (C) Treatment of PR8-infected A549 cells with LY294002 (lane 2), TCL1-*Akt-in* (lane 4), or TCL1b-*Akt-in* (lane 5) efficiently inhibited phosphorylation of Akt along with decreased levels of NS1 phosphorylation. (D) Treatment of PR8-infected A549 cells with LY294002 (lane 2), TCL1-*Akt-in* (lane 4), or TCL1b-*Akt-in* (lane 5) efficiently inhibited viral replication in plaque assays. The pictures of the plaque phenotypes of each treatment were shown underneath.

encompassing the βA strand of TCL1), and "TCL1b-*Akt-in*" (a TCL1b-based Akt inhibitor). Both TCL1- and TCL1b-*Akt-in* inhibited Akt kinase activity and, hence, suppressed proliferation of cancer cells [20–22]. In order to suppress Akt kinase activity, we used TCL1- or TCL1b-based *Akt-in*, a peptide Akt kinase inhibitor [20,21].

LY294002, a classical PI3K inhibitor, is known to compete for the ATP-binding site of the catalytic domain of lipid kinases. LY294002 treatment efficiently inhibited phosphorylation of Akt as well as that of NS1. Treatment of PR8-infected A549 cells with TCL1-*Akt-in* (or TCL1b-*Akt-in*), efficiently inhibited phosphoryla-

tion of both Akt and NS1 (Fig. 1C). In contrast to the levels of total Akt, which remained unchanged after treatment of cells with LY294002, TCL1-*Akt-in*, or TCL1b-*Akt-in*, the levels of NS1 expression appeared to be decreased, which may affect the decreased levels of the phospho-NS1 observed in this experiment. Moreover, treatment of PR8-infected A549 cells with LY294002, TCL1-*Akt-in*, or TCL1b-*Akt-in* efficiently inhibited viral replication in plaque assays (Fig. 1D)

3.3. Inhibition of Akt kinase activity by TCL1- or TCL1b-based peptide inhibitors (Akt-in) efficiently suppresses viral replication in a dose- and time-dependent manner

The influenza virus is considered to evade both innate and adaptive immunity via the NS1 protein, during which viral NS1 is known to activate PI3K signaling pathway as one of the major downstream effectors that enhances efficient replication [11–13]. Recent studies suggested that cellular signaling pathway inhibitors may hold promise as antiviral therapy against influenza [15,25,26]. These observations prompted us to examine whether inhibition of Akt kinase activity in the virus-infected host cells could represent a potential therapeutic target against influenza viral infection.

Treatment of PR8-infected A549 cells with increased amounts of TCL1-*Akt-in* (Fig. 2A) or TCL1b-*Akt-in* (Fig. 2B) correlated inversely with the levels of phosphorylation of Akt and NS1, in a dose-dependent manner. It is noteworthy that the levels of endogenous Akt did not decrease with even with a TCL1-*Akt-in* concentration of up to 50 μM. However, total NS1 levels seemed decrease after treatment with 50 μM of TCL1- or TCL1b-*Akt-in*, which may also have contributed to the decreased levels of the phosphorylated form of NS1 that were observed in this experiment. The decreased NS1 expression was consistent with that observed after treatment of MK2206, an allosteric inhibitor of Akt [19]. To further evaluate the effect of viral replication observed in the dose escalation treatment, we conducted viral plaque assays. We found that indeed, treatment with increased amount of TCL1-*Akt-in* (Fig. 2C) or TCL1b-*Akt-in* (Fig. 2D) efficiently inhibited viral replication in a dose-dependent manner.

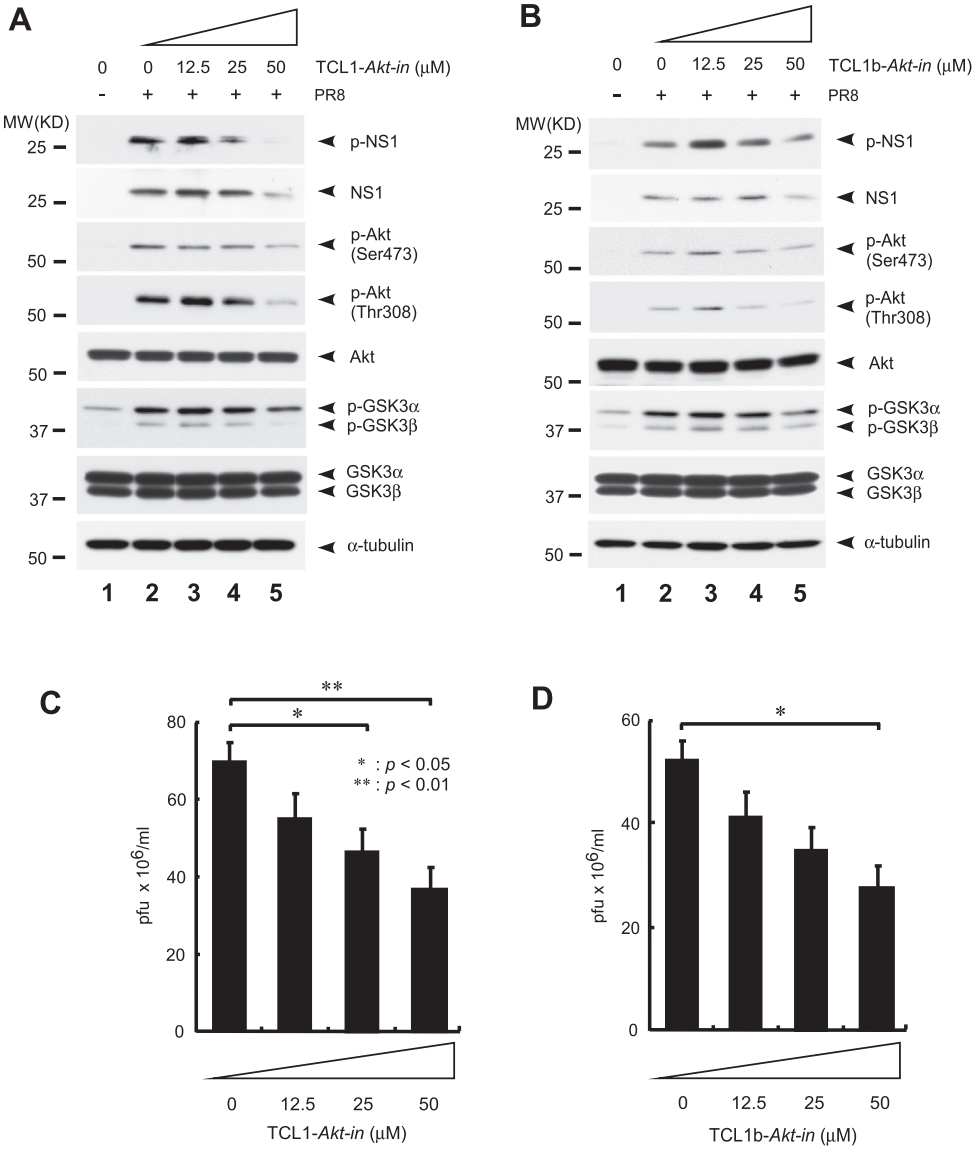

Fig. 2. (A and B) Treatment of PR8-infected A549 cells with increased amount of TCL1- *Akt-in* (panel A) or TCL1b-*Akt-in* (panel B) correlated inversely with the levels of phosphorylation of Akt (third panel from the top) and NS1 (top panel). (C and D) Increased amounts of TCL1-*Akt-in* (panel C) or TCL1b-*Akt-in* (panel D) treatment efficiently inhibited virus replication in plaque assays.

Since PI3K–Akt signaling is known to play a role in various stages of influenza virus infection [13,18], we next examined the effect of TCL1- or TCL1b-*Akt-in* for during long-term treatment, to evaluate the effect of phosphorylation of Akt and NS1 in the PR8-infected A549 cells, using time-course experiment. Treatment of PR8-infected A549 cells with TCL1-*Akt-in* or TCL1b-*Akt-in* efficiently inhibited the levels of phosphorylation of Akt at Ser473, Thr308, and NS1 by 24 h, 48 h, and 72 h after PR8 infection (Fig. 3A). The inhibitory effect of the kinase activity in the virus infected host cells correlated markedly with inhibition of influenza virus replication at 24 h, 48 h, and 72 h after the PR8 infection in the plaque assays (Fig. 3B). These observations indicated that TCL1- and TCL1b-based inhibitors can inhibit both the early phase (virus attachment and/or viral entry) and late phase of PI3K-activation events (including production of inflammatory cytokines such as IL-6 or IL-8) [13].

3.4. Inhibition of Akt kinase activity blocks viral entry without inhibiting cytokine production in the host cells

Influenza viral infection is known to increase inflammatory reaction, which promotes production of IL-6 or IL-8 [26–29]. In order to evaluate the nature of inhibitory effect observed by treatment of PR8-infected host cells with TCL1-*Akt-in* or TCL1b-*Akt-in*,

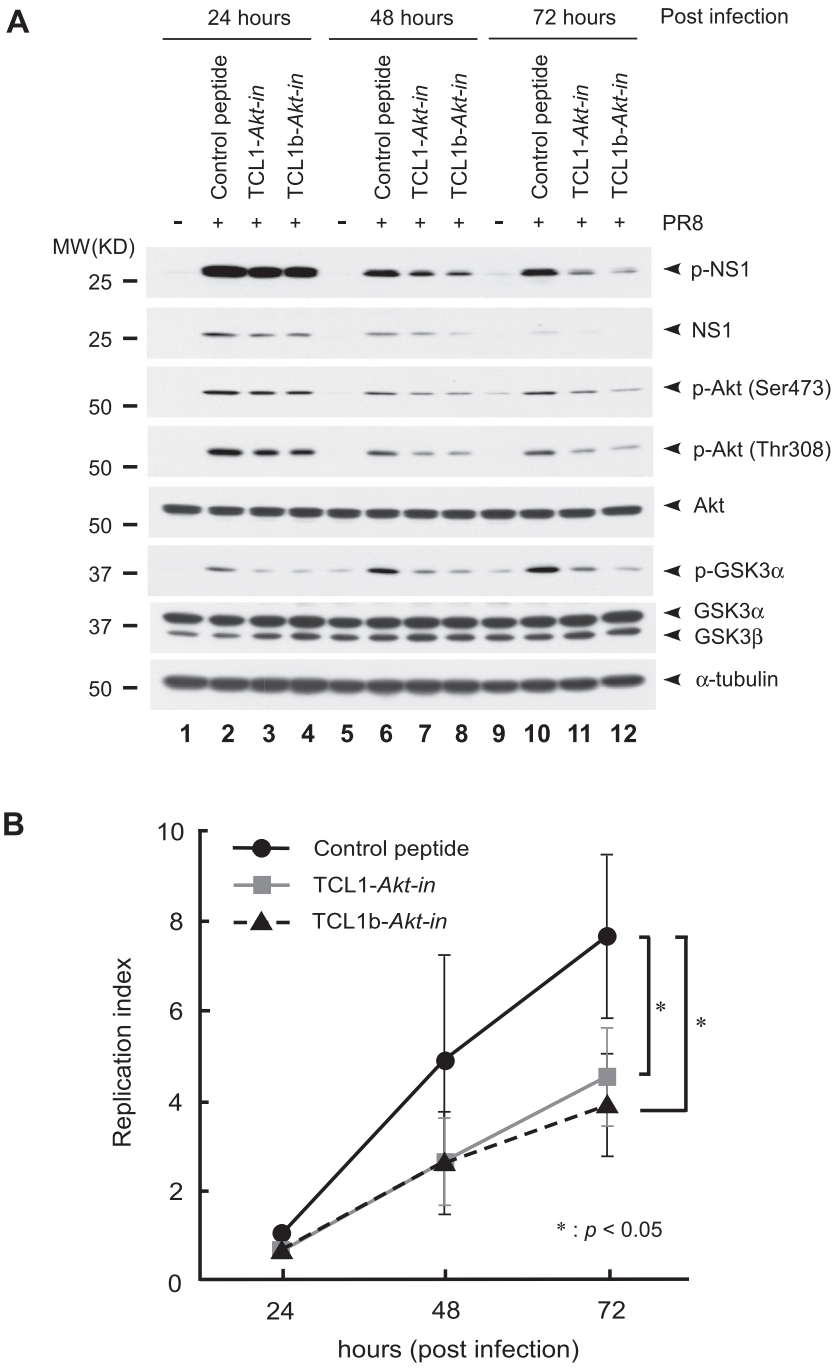

Fig. 3. (A) Treatment of PR8-infected A549 cells with TCL1-*Akt-in* or TCL1b-*Akt-in* efficiently inhibited the levels of phosphorylation of Akt at Ser473 (third panel from the top), Thr308 (fourth panel from the top), and NS1 (top panel) at 24 h after infection of cells with PR8 influenza virus (lanes 1–4), 48 h (lanes 5–8), and 72 h (lanes 9–12). (B) Treatment of PR8-infected A549 cells with TCL1-*Akt-in* or TCL1b-*Akt-in*, but not control peptide, efficiently inhibited the replication of influenza virus at 24 h, 48 h, and 72 h after infection of cells with PR8 influenza virus in plaque assays.

we next measured the production of IL-6 and IL-8 in the supernatant of PR8-infection of A549 cells. PR8 infection of A549 cells markedly increased the production of both IL-6 and IL-8 approximately two- to threefold. Treatment of these cells with LY294002, a PI3K inhibitor, suppressed the production of IL-6 or IL-8, both of which are considered to be important inflammatory cytokines for viral infection [26–29]. However, the culture supernatant from TCL1-*Akt-in*- or TCL1b-*Akt-in*-treated PR8-infected A549 cells showed no inhibition of IL-6 (Fig. 4A) or IL-8 production (Fig. 4B). This observation suggested that, in contrast to LY294002, inhibition of viral replication by TCL1-*Akt-in* or TCL1b-*Akt-in* is not due to the inhibition of production of inflammatory cytokines such as IL-6 or IL-8. The discrepancy is possibly due to the off-target effect of LY294002, since LY294002, a classical PI3K inhibitor, possibly inhibits additional regulatory molecules other than Akt as downstream effectors of PI3K, hence suppressing cell growth and protein translation by inhibiting p70S6 kinase [1,2,4]. In contrast, *Akt-in*, which specifically inhibits association of PtdIns with Akt, has potent inhibitory effects specific to Akt kinase, one of the major downstream effector molecules of PI3K [20].

To further examine the nature of the inhibition of viral replication by TCL1-*Akt-in* or TCL1b-*Akt-in*, we next investigated the mechanisms underlying the dose- and time-dependent inhibition observed in the plaque assays. We used nucleoprotein, a multi-

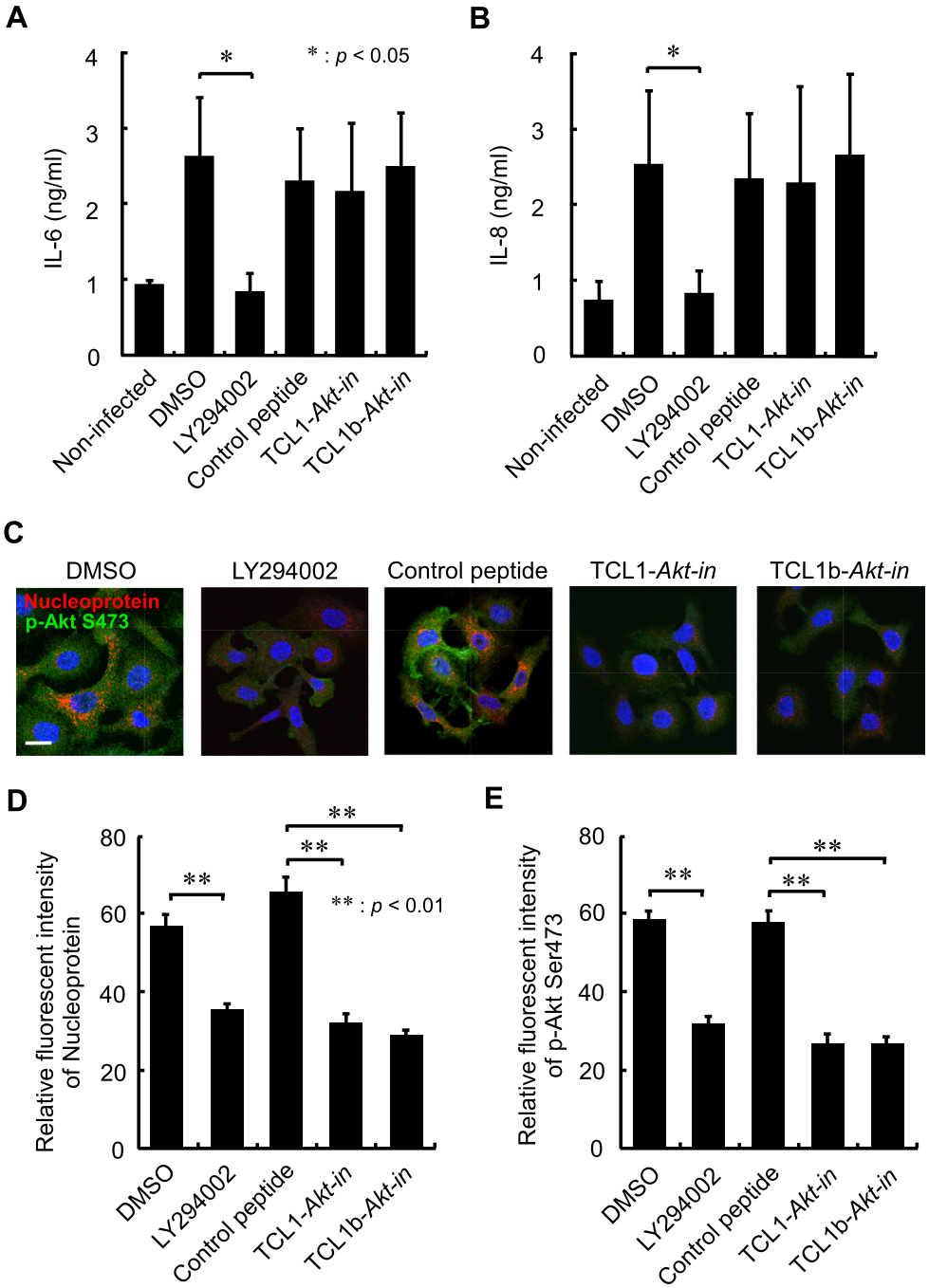

Fig. 4. (A) Quantification of IL-6 in the culture supernatants of A549 cells infected with PR8 at a MOI = 0.1, used for plaque assays. (B) Quantification of IL-8 in the culture supernatants of A549 cells infected with PR8 at a MOI = 0.1, used for plaque assays. (C) Ninety minutes after PR8-infection of A549 cells were stained with anti-nucleoprotein or anti-phospho-Akt antibodies and examined by confocal microscopy to determine the uptake of influenza virus. (D) Quantification of nucleoprotein from panel C ($n = 10$). (E) Quantification of phosphorylated Akt from panels C ($n = 10$).

functional RNA-binding protein that is pivotal to virus replication, as a detection marker for influenza viral infection [30]. Consistent with the immunoblot results shown in Fig. 1A, upon double positive immunostaining of both anti-nucleoprotein and anti-phospho-Akt antibodies by confocal microscopy, PR8-infected A549 cells showed a marked increase in phosphorylation of Akt (Fig. 4C). Treatment of cells with TCL1- or TCL1b-*Akt-in* decreased the levels of phospho-Akt, which appeared to be correlated well with the decreased levels of influenza virus uptake, as determined by the red signal of the nucleoprotein (Fig. 4C–E). Since PI3K–Akt signaling pathways in host cells can be activated by influenza NS1 [7,11,23,24], the inhibition of viral uptake/entry may account for the decreased expression of influenza virus NS1, hence inhibited the levels of phospho-Akt in PR8-infected A549 host cells.

4. Discussion

In this study, we showed treatment of cells with either "TCL1- or TCL1b-based *Akt-in*" [20,21], efficiently suppressed Akt kinase activity while decreasing the levels of phosphorylated NS1; this, in turn, inhibited viral entry and replication without inhibiting the production of inflammatory cytokines.

For replication and transcription of the influenza virus genome, various host cell-derived factors are required such as PI3K–Akt pathways [25,26]. Hence, Akt inhibitors are an attractive target for therapy against human viral infection. Several attempts have been made to develop Akt inhibitors [2]. We have previously identified a peptide, named "*Akt-in*", which interacts with Akt and specifically inhibits its kinase activity [20,22]. The TCL1- or TCL1b-based inhibitor "*Akt-in*" effectively inhibited the Akt kinase activities of PR8-infected host cells, and consequently suppressed viral uptake and replication without inhibiting the production of inflammatory cytokines. Our observations are consistent with a recent report by Deniscova that MK2206, an allosteric Akt inhibitor, inhibited NS1 expression and, hence, prevented H1N1 influenza infection [19]. We showed that treatment with the Akt inhibitor suppressed GSK3 phosphorylation levels (Figs. 2 and 3); this was in agreement with the report that GSK3-β, an Akt substrate [31], is a key regulatory molecule for influenza viral entry [11,14,25]. Activated Akt phosphorylates GSK3, reducing its activity, which lead to enhance production of glycogen [31].

H1N1 avian influenza virus-induced disease has emerged and now appears to be present in a sufficient number of countries to be considered a major global health concern. Multiple cellular signaling pathways are involved in influenza virus replication [14,15,18,26]. In addition to direct involvement in tumorigenesis by genetic alterations of human cancers, the PI3K–Akt network also underlies the clinical manifestation of various stages of viral infection [2,13,16]. By effectively activating the PI3K–Akt network, virally infected cells can presumably avoid their own virus-induced death. Inhibiting anti-apoptotic PI3K–Akt signaling may, thus, allow scavengers to control the various stages of host cell-to-cell infectious processes [9,19]. The PI3K–Akt–mTOR pathway has also been suggested to play a role in the regulation of Macroautophagy [32], which is involved in various stages of viral infections by controlling the innate immunity [33–35]. However, in our preliminary experiments using Akt inhibitors did not alter the induction of autophagy in PR8-infected A549 cells, excluding the possibilities of the involvement of autophagy in this process.

In this study, we showed that the TCL1- and TCL1b-based inhibitors effectively inhibited Akt kinase activity in PR8-infected host cells, and consequently effectively suppressed viral entry, NS1 expression, without affecting the cytokine production. These observations supported that TCL1- and TCL1b-based inhibitors can inhibit both early phase (viral entry) and late phase of viral infection events (viral genome replication). In contrast to the anti-viral pharmacological reagents targeting the influenza viral particles, inhibition of Akt kinase activity by *Akt-in* contributed to suppress multiple steps of influenza virus infection without affecting the immune reaction of the host cells. Thus, combination usage of specific inhibitors for Akt with anti-viral pharmacological reagents may have a benefit against influenza infection, a life threatening human disease. The observation will facilitate the design of specific inhibitors for Akt, a core intra-cellular survival factor underlying influenza infections.

Acknowledgments

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