Influenza Virus Induces Apoptosis via BAD-mediated Mitochondria Dysregulation

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Abstract

Influenza virus infection results in host cell death and major tissue damage. Specific components of the apoptotic pathway, a signaling cascade that ultimately leads to cell death, are implicated in promoting influenza virus replication. BAD is a cell death regulator that constitutes a critical control point in the intrinsic apoptosis pathway, which occurs through the dysregulation of mitochondrial outer membrane permeabilization and the subsequent activation of downstream apoptogenic factors. Here we report a novel pro-viral role for the pro-apoptotic protein BAD in influenza virus replication. We show that influenza virus-induced cytopathology and cell death are considerably inhibited in BAD knockdown cells and that both virus replication and viral protein production are dramatically reduced, which suggests that virus-induced apoptosis is BAD-dependent. Our data showed that influenza viruses induced phosphorylation of BAD at residues S112 and S136 in a temporal manner. Viral infection also induced BAD cleavage, late in the viral lifecycle, to a truncated form that is reportedly a more potent inducer of apoptosis. We further demonstrate that knockdown of BAD resulted in reduced cytochrome c release and suppression of the intrinsic apoptotic pathway during influenza virus replication, as seen by an inhibition of caspases-3, caspase-7 and PARP cleavage. Our data indicate that influenza viruses carefully modulate the activation of the apoptotic pathway that is dependent on the regulatory function of BAD, and failure of apoptosis activation resulted in unproductive viral replication.
Introduction

Apoptosis induced during influenza virus infection is a major contributing factor to cell death and tissue damage (3, 12, 14, 22, 34). Studies with the 1918 pandemic virus in macaques showed that activation of the apoptotic pathway was a source of tissue damage during infection (2, 4).

Apoptosis, or programmed cell death, is an important cellular signaling response often observed after viral infections. Apoptosis is the process whereby individual cells undergo regulated self-destruction in response to a variety of stimuli. In mammalian cells, the apoptotic pathway can be divided into two signaling cascades: the extrinsic and the intrinsic apoptotic pathways (9). Induction of the extrinsic apoptotic pathway involves the stimulation of death receptors belonging to the tumor necrosis factor receptor (TNFR) family, such as Fas and the tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) (9). The intrinsic apoptotic pathway acts through the mitochondria upon activation, and this signaling process is highly regulated by the Bcl-2 family of proteins (18).

The Bcl-2 protein family consists of both anti- and pro-apoptotic members that form a critical decision point within a common cell-death signaling pathway (18). The delicate balance between anti- and pro-apoptotic protein activities dictates whether a cell will succumb to an apoptotic stimulus or not (1). Our current understanding of the canonical apoptosis mechanism involves activation of the signaling transduction pathway by an external cell death stimulus. The cell death signal gets transmitted through pro-apoptotic factors such as Bax and Bak that go on to inflict mitochondrial damage and cytochrome c release (21). Inhibition of apoptosis is mainly due to the activities of Bcl-2 and Bcl-xL, which sequester Bax and prevent it from inflicting mitochondrial damage (13). Bcl-2 and Bcl-xL are well known targets of the pro-apoptotic protein...
Bcl-2 antagonist of cell death (BAD), which specifically blocks the activity of both anti-
apoptotic factors by forming heterodimeric complexes with either of the two proteins and
displacing Bax (38, 40).

Apoptosis has long been considered a host cell defence response because various pathogenic
viruses express anti-apoptotic proteins to prevent this cellular response (23). However, there is
now accumulating evidence to strongly suggest a number of viruses may manipulate the cell
death signaling pathway to promote viral replication, including influenza viruses (20, 22, 26, 27,
29, 30, 32, 36, 37). Influenza virus infection resulted in the up-regulation of pro-apoptotic factors
such as TRAIL, and the death receptor Fas and its ligand FasL, reportedly via NF-κB induction
(36). Blockage of TRAIL and Fas signaling with soluble monoclonal antibodies to their
respective receptors resulted in significant reduction of viral titer (36). Pro-apoptotic factors also
play critical pro-viral roles for other viruses such as HIV-1. A study reported that HIV-1 virus
production was enhanced upon expression of FasL (35).

Several lines of evidence have revealed both an agonistic and an antagonistic role for the
Bcl-2 family in influenza virus propagation. Early studies demonstrated that ectopically
overexpressed anti-apoptotic protein Bcl-2 resulted in impaired virus production and inhibition
of influenza virus-induced apoptosis (12, 30). However, pro-apoptotic proteins Bak and Bax
have been reported to have conflicting roles. One study suggested Bak has an anti-viral role in
influenza replication and was downregulated upon viral infection (26). Paradoxically, Bax
activation was necessary for efficient influenza virus propagation (26). Thus, it is likely that only
a subset of the pro-apoptotic Bcl-2 family members positively contribute to influenza virus
infection.
BAD is a cell death regulator that constitutes a critical control point in the intrinsic pathway of apoptosis, which occurs through the dysregulation of the mitochondrial outer membrane permeabilization and the subsequent release of apoptogenic factors. We hypothesize that BAD plays an important regulatory role in influenza virus’ induction of the intrinsic apoptosis signaling cascade. Here we report a novel pro-viral role for the pro-apoptotic protein BAD in influenza virus replication. We show that influenza virus-induced cytopathology and cell death is considerably inhibited in BAD knockdown cells and that both virus replication and viral protein production are dramatically reduced in BAD-deficient cells. Knockdown of BAD resulted in reduced cytochrome c release and suppression of the intrinsic apoptotic pathway during influenza virus replication.

**Materials and Methods**

**Cells and virus.** HEK-293T cells and A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1X non-essential amino acids (Gibco®, Invitrogen), 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine. Influenza virus strains A/New York/55/2004 (H3N2) (NY55), A/Puerto Rico/8/1934 (H1N1) (PR8) and A/California/07/2009 (H1N1) (SOIV) were grown in 10-day old embryonated chicken eggs and titered on MDCK cells.

**Lentivirus Packaging and Transduction.** *E. coli* clones containing individual human shRNAmir, or a non-targeting shRNAmir control, in pGIPZ plasmids were obtained from Open Biosystems. These *E. coli* clones were cultured in 2× LB broth (low salt; 2%(w/v) LB-Broth-Lennox; 1%(w/v) Peptone; 0.5%(w/v) Yeast Extract; 100 μg/ml ampicillin) at 37°C overnight.
with shaking at 250 rpm. Plasmids were isolated with QIAGEN Maxiprep kit following manufacturer’s protocol. Individual shRNAs were packaged into lentivirus particles by cointransfection of each shRNAmir-pGIPZ with pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260, respectively) in HEK-293T cells at a ratio of 2:2:1, respectively, with transfection reagent Arrest-In (Open Biosystems). Lentivirus-containing supernatants were harvested at 48 hours (h) and 72 h post-transfection. After the last harvest time point, supernatants were centrifuged at 64,000 ×g for 1.5 h at 4°C, and lentivirus-containing pellets were resuspended in serum-free 1× Dulbecco’s modified Eagle’s medium (DMEM). Lentivirus particles were titered on A549 cells and GFP-expressing cell colonies enumerated with Axio Observer.Z1 fitted with EC Plan-NEOFLUAR 10×/0.3 Ph1 M27 objective (Carl Zeiss MicroImaging GmbH, Germany). Stable KD A549 cells were produced by transducing with lentivirus at an MOI of 0.5. At 72 h post-transduction, 3 ug/ml puromycin (Sigma) was added to the media. Cells were passaged over a 2 week period in puromycin-supplemented completed 1× DMEM media to select transductants before they were infected with virus (described under Influenza Virus Infection). shRNA sequences are listed in Table 1.

**siRNA Transfection.** A549 cells were treated with 25 nM of each of 4 ON-Targetplus siRNA (Dharmacon) targeting host gene or an ON-Targetplus non-targeting siRNA control. siRNAs were introduced into cells with Lipofectamine RNAiMAX (Invitrogen). 1X serum-free Opti-MEM media (Invitrogen) was used to dilute the siRNAs and RNAiMAX separately; the two diluants were combined and incubated at room temperature for 20 minutes. After incubation, the transfection mixture was added to the cells. Each cell set was re-treated with the same siRNA 24
h later. After an additional 24 h (48 h after initial transfection), cells were infected with virus. siRNA sequences are listed in Table 1.

**Influenza Virus Infection and Plaque Assay.** Sets of transduced or transfected A549 cells were infected with influenza virus strains A/New York/55/2004(H3N2) at an MOI of 1 (shRNA) or 0.1 (siRNA) PFU/cell; or with A/PR/8/34(H1N1) or SOIV at an MOI of 0.1. Cell monolayers were washed twice with 1× phosphate buffered saline (PBS; 137 mM NaCl, 0.3 mM KCl, 0.8 mM Na₂HPO₄, 0.1 mM KH₂PO₄) and infected with viruses diluted in gel saline (137 mM NaCl, 0.2 mM CaCl₂, 0.8 mM MgCl₂, 19 mM HB₃O₃, 0.1 mM Na₂B₄O₇, 0.3%(w/v) gelatin). At 72 hpi, supernatants were harvested and virus yield was titrated by plaque assay on MDCK cells. MDCK cell monolayers were washed twice with 1× PBS, infected with 1:10 serial dilutions of viruses in gel saline, and overlaid with 0.6% type-I agarose (Sigma Aldrich) for 3 days. All influenza virus infections occurred at 35°C in 5% CO₂ humidified environment, including the plaque assay. For western and immunofluorescence assays, cells were infected with NY55 at MOI 1 and processed at 24 hpi.

**Western Blot.** At specific time points after influenza virus infection, and as indicated in the respective figures, whole cell lysate was obtained by lysing cells in RIPA buffer (50mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% SDS) with complete protease inhibitor (Roche). 20 μg of lysate was solubilized in 1X protein sample buffer (0.3 M Tris, pH 6.8; 50% Glycerol; 0.3 M SDS; 5 mM DTT; 10 mM Bromophenol blue) and subjected to electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE), transferred to Immobilon-P PVDF membranes (Millipore), and blotted with mouse monoclonal α-NS1 (3F5), mouse
monoclonal α-NP (F26-9 (39), gift from Dr. Mingyi Li), rabbit polyclonal α-HA (Rockland), mouse monoclonal α-BAD (C-7, Santa Cruz), mouse monoclonal α-cleaved caspase-3 (Asp175, R&D Systems), rabbit monoclonal α-phospho-BAD (Ser136, Cell Signaling), mouse monoclonal α-phospho-BAD (Ser112, Cell Signaling), rabbit polyclonal α-cleaved PARP (Asp214, Cell Signaling), rabbit monoclonal α-cytochrome c (Cell Signaling), rabbit polyclonal α-cleaved caspase-7 (Asp198, Cell Signaling), or rabbit polyclonal α-beta-actin (Cell Signaling) antibodies. Cytoplasmic lysate for caspase immunoblot was obtained by lysing cells in Caspase Lysis buffer (20 mM Tris-Hcl, pH 7.5; 0.5% NP-40, 0.5 mM PMSF, 100 μM β-glycerol-3-phosphate; protease inhibitor cocktail (Roche)). 40 μg of lysate was solubilised in 1× protein sample buffer and electrophoresed on 15% SDS-PAGE and transferred to Immobilon-P sq PVDF membranes (Millipore). Blots were subjected to secondary α-rabbit or α-mouse polyclonal antibody conjugated with HRP (Cell Signaling) and detected with in-house enhanced chemiluminescence (ECL) reagent. Blot image and protein band quantification was obtained with Alpha Innotech FluorChem® Q Imaging System and processed using Adobe® Photoshop®.

**Caspase-Glo 3/7 Assay.** Caspase-3/7 activity was detected using Caspase-Glo® 3/7 assay (Promega), according to manufacturer’s protocol. 5000 cells were seeded per well in 96-well, white-walled plates. Cells were infected with NY55 virus at MOI 1. Staurosporine was used as a positive control for caspase activity. Cells were treated with 1 μM staurosporine for 24 h. Caspase substrate was added to each well at 72 hpi and 24 h post-treatment for staurosporine. Luminescence was detected with a BioTek Synergy™ 4 plate reader and data was processed with Gen5™ software.
Real-Time PCR. Total mRNA was isolated using RNeasy Mini Kit (QIAGEN) according to manufacturer’s protocol. 500 ng of purified mRNA was used to generate cDNA with random hexamer primers (Applied Biosystems) and SuperScript® II Reverse Transcriptase (Invitrogen) according to manufacturer’s protocol. qRT-PCR reaction mix (25 µl) consisted of: 12.5 µl of SYBR® Green PCR Master Mix (Invitrogen), 0.5 µl cDNA template, and 1 µl of each of 100 µM forward and reverse primers (Table 2). Reactions were run in duplicate on Applied Biosystems 7300 Real-Time PCR System. The cycling conditions were as follows: 50°C for 2 min., 95°C for 2 min., and 50 cycles of (95°C for 15 sec. and 60°C for 30 sec.). Cₜ values were normalized to 18S rRNA control and compared to non-targeting (“non-targeting”) sh/siRNA negative controls.

Cell Viability. Cell viability was determined using Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer’s protocol or trypan blue exclusion assay. For trypan blue exclusion assay, ~ 1x10⁶ infected or uninfected cells were stained with 20 µl of trypan blue solution and ~ 14 µl of the stained cells were placed on a hemocytometer. A total of 200 cells were counted and the percentage of viable cells was calculated with the following formula:

\[
\text{% viable cell} = \frac{\text{(total number of cells counted)} - \text{(total number of dead cells)}}{\text{total number of cells counted}} \times 100
\]

Statistics. Statistical analysis was calculated using ANOVA and student’s t-test.

Results
Influenza virus-induced cytopathology and cell death is inhibited in BAD-deficient cells.

Influenza virus killing of host cells is known to occur through the activation of the apoptotic signaling pathway (12, 26). BAD is an important regulator of anti-apoptotic Bcl-2 and Bcl-xL proteins. Its blockage of the latter two factors defines the fate of the host cell towards apoptosis. In order to determine the effect of BAD on influenza virus replication, we generated BAD knockdown (KD) cells. A549 cells were treated with shRNA, or siRNA oligomers, that target BAD transcripts to create stable or transient knockdown cells, respectively. BAD-shRNA KD cells were infected with A/New York/55/2004 (H3N2) virus (NY55), A/Puerto Rico/8/1934 (H1N1) virus (PR8) or the 2009 pandemic swine-origin influenza virus (SOIV) (A/California/07/2009). Infected cells were examined visually for demonstration of cytopathic effect (CPE), which phenotypically manifests as rounding up and detachment of infected cells as well as abnormal cellular structural morphology. Non-transduced cells and non-targeting shRNA-transduced controls infected with any tested influenza virus subtype (NY55, SOIV, or PR8) showed extensive CPE indicative of virus-induced cytopathology (Fig. 1 A). In contrast, there was no observable CPE in influenza virus-infected cells that had been transduced with BAD-specific shRNAs. To ensure that our observation was not biased by artefacts derived from the use of shRNA constructs and lentivirus transductions, and to determine if the same observations would manifest in transient KD, we used siRNA duplexes to knockdown BAD in another set of A549 cells and infected these with the same virus subtypes. Comparable results were observed with siRNA-treated cells (Fig. 1 B). This lack of CPE development in virus-infected BAD KD cells suggests inhibition of the virus’ capacity to induce cytopathology in BAD-deficient cells.
CPE development does not necessarily correlate with cell viability; therefore, a more quantitative means of determining cell viability was carried out by measurement of mitochondrial activity (WST-1) and by trypan blue exclusion. Both assays showed greater viability of virus-infected BAD KD cells compared to non-targeting shRNA and untransduced cell controls. In NY55 infected cells, 100% of the BAD KD cells survived viral infection, as measured by mitochondrial activity, and over 93% of cells survived PR8 infection; this is compared to controls with less than 65% survival in non-targeting and untransduced cells ($P < 0.001$) (Fig. 1 C).

Similarly, greater than 76% of the BAD KD cells survived influenza virus infection at 72 hpi for all three virus subtypes (Fig. 1D), as measured by trypan blue exclusion, compared to less than 40% of the cells surviving infection in the non-targeting and untransduced controls.

Efficient KD of endogenous BAD was validated by quantitative real-time PCR (qRT-PCR) and Western blot to ensure the effectiveness of the shRNA or siRNA treatment. BAD mRNA KD was confirmed by qRT-PCR for both shRNA and siRNA treatments (Fig. 2 A, B). Western blots for endogenous BAD protein supports the real-time PCR results (Fig. 2 C, D). BAD protein was significantly reduced for both shRNA species (Fig. 2 C) as well as for all 4 siRNA oligomers tested (Fig. 2 D). Treatment of A549 cells with shRNA or siRNA alone did not affect cell viability as determined by the WST-1 cell proliferation assay (Fig. 2 E, F). For proper comparison of cell viability and BAD knockdown, both cell viability and BAD mRNA isolation was carried out at the same time—2 weeks after shRNA transduction, which allows for the establishmentment of a stable knockdown cell line, and 48 h after the initial siRNA transfections.
The greater percentage of cell survival, and lack of CPE development despite viral infection, suggests BAD is involved in promoting virus-induced cytopathology and cell death. Therefore, our next objective was to assess the capacity for influenza viruses to induce apoptosis in BAD KD cells.

BAD knockdown in A549 cells reduced influenza virus replication of different virus subtypes. A number of studies have reported the importance apoptosis plays in promoting efficient influenza virus replication (36, 37). Given the critical pro-apoptotic nature of BAD and the lack of CPE and cell death development we observed in BAD KD cells, we hypothesize that influenza virus replication is suppressed in BAD-deficient cells. To explore this possibility, stable BAD KD and non-targeting shRNA A549 cells were infected with NY55, and virus replication was followed over a 72 h period. Virus progeny yield was also determined for PR8 and SOIV. Virus progeny production was titered by plaque assay on MDCK cells.

The initial rounds of virus replication in shRNA BAD KD cells and shRNA non-targeting control (shNSi) cells from 0 hpi to 12 hpi were comparable (Fig. 3A). However, subsequent viral replication after 12 hpi was less efficient in BAD KD cells compared to the control cells (Fig. 3A). NY55 production was significantly reduced to less than 37% and 15% of the non-targeting shRNA control with BAD-specific 202976-shRNA and 15289-shRNA, respectively (Fig. 3 B, P = 0.011). Reduction of viral titer was much more dramatic with SOIV and PR8, which replicated to only about 1% of levels seen in the non-targeting control (Fig. 3 D, P < 0.001).

To ensure that a reduction in viral replication was not due to the effect of shRNA and/or lentivirus treatment, we repeated the infections in siRNA transiently transfected A549 cells.
Cells were sequentially treated twice with each of four distinct siRNAs that target BAD (plus a non-targeting siRNA control) 24 h apart, and after a further 24 h, were infected with NY55, PR8, or SOIV influenza viruses. Virus titer was determined at 72 hpi.

Replication of all three virus strains was dramatically reduced in siRNA BAD KD cells (Fig. 3 C, E). NY55 production was reduced to less than 24% of the non-targeting control by all four siRNA species (Fig. 3 C, P < 0.001). SOIV and PR8 titers were less than 10% and 11% of the controls, respectively; this was slightly lower than that detected for NY55 titer, which was ~19% of the control (Fig. 3 E, P < 0.001). Our observation with siRNA confirms the results determined with the infection of lentivirus-mediated BAD shRNA knockdown. These data strongly indicate that BAD is a significant host factor required for efficient influenza virus replication. Of significance is the applicable role for BAD to the lifecycles of different influenza virus subtypes.

**Influenza virus protein production is inhibited in BAD knockdown cells.** Influenza virus-induced cell death occurs late in the viral lifecycle; as late as 15 hpi has been reported (41). The lack of cell death coupled to significant reduction in viral replication in BAD KD cells raises the question of whether influenza virus replication in BAD-deficient cells may be inhibited early in the viral lifecycle. In order to explore this, we carried out western blot assays on whole cell lysates of stable shRNA BAD KD and non-targeting shRNA control cells that were infected with NY55. Viral protein production was determined at specific time points over a 72 h period. The membrane was probed for influenza virus proteins NS1, NP, and HA. The NS1 mouse monoclonal antibody was generated and characterized in our lab (Rahim et al., submitted), and the characterization of the mouse monoclonal NP antibody was previously described (39). In the
stably expressing non-targeting shRNA control cells, NS1, NP, and HA viral proteins were detected as early as 4 hpi, and strongly detected at 8 hpi onwards (Fig. 4A, left column). Protein bands were faintly detected as early as 0 hpi for the HA viral protein. Since HA is incorporated into virion particles, the early detection could be due to the infecting viral population initially introduced. In contrast, the production of all three viral proteins were clearly reduced in infected BAD KD cells (Fig. 4A, right column). Densitometric analysis of the viral protein bands showed an average of 5-fold and 4-fold reduction of NP and HA protein, respectively, in BAD KD cells compared to the non-targeting (shNSi) shRNA control (Fig. 4B, D). NS1 protein production between BAD KD cells and non-targeting shRNA control is comparable from 8 hpi to 22 hpi but NS1 protein level dramatically dipped lower in BAD KD cells between 24 - 72 hpi, with an average of 3-fold reduction in BAD KD cells (Fig. 4C). The results suggest that BAD KD significantly reduces the efficiency of viral replication.

**Influenza virus induces phosphorylation and cleavage of BAD.** BAD’s capacity to bind and neutralize anti-apoptotic proteins is inhibited upon phosphorylation (6). Given that our results showed BAD as a valuable cellular factor required for influenza virus replication, we suspected that viral replication might affect BAD activity and how BAD is regulated. We infected A549 cells with NY55 and harvested samples at specific time points post-infection. The samples were subjected to Western blot analysis and probed for BAD phosphorylation at sites S112 and S136. Total BAD was also determined.

Our results showed that influenza virus infection induces BAD phosphorylation at both S112 and S136 but in a sequential manner (Fig. 5). Phosphorylation at S112 occurred as early as 14 hpi, with most intense bands observed at 20-22 hpi, and was gradually reduced after 24 hpi.
Phosphorylation at S136 wasn’t detected until 20 hpi; the signal was maintained until 48 hpi but then decreased by 72 hpi. Total BAD, including unphosphorylated BAD, showed a gradual increase towards the late time points. Interestingly, we detected the smaller cleaved form of BAD at 48 hpi and 72 hpi, with the latest time point showing the greatest cleavage of BAD (Fig. 5).

All these modifications observed during influenza virus infection were not detected in uninfected (mock) controls. Our results suggest that influenza viruses tightly control BAD activity via phosphorylation and cleavage to regulate the intrinsic apoptotic signaling cascade.

**BAD knockdown suppresses cytochrome c release during influenza virus infection.** Given that BAD is a well-known regulator of the mitochondria-dependent apoptosis pathway (6, 15), we wanted to determine whether a deficiency in BAD would suppress influenza virus’ capacity to induce this signaling pathway during infection. Cytochrome c release from the mitochondria as a result of the organelle’s dysregulation is a hallmark of the intrinsic apoptosis signaling pathway (15). To address this issue, we carried out a cytochrome release assay, which briefly involved the following steps: infection of BAD KD and non-targeting control cells with NY55, cells were harvested at specific time points post-infection, and lysed to obtain the cytosolic and organelle fractions (labeled as mitochondrial pellet in Fig. 6).

We observed an increase in the release of cytochrome c into the cytosol of infected non-targeting control at 20 hpi to 48 hpi relative to the uninfected (mock) samples (Fig. 6, row 1). As expected, the corresponding mitochondrial pellet of the infected non-targeting control showed a decrease in cytochrome c at 20 hpi to 48 hpi (Fig. 6, row 5). We did not observe a similar increase in cytochrome c release into the cytosol of infected BAD KD cells (Fig. 6, row 2), and the amount of cytochrome c in the mitochondrial pellets were similar for the infected and...
uninfected BAD KD cells (Fig. 6, row 6). These data suggest that influenza viruses induce mitochondria dysregulation late in the virus replication cycle, as observed by cytochrome c released into the cytosol of infected cells, and that BAD is required for virus-induced dysregulation of the mitochondria.

BAD is required for efficient induction of caspase activity after influenza virus infection. Influenza virus infection results in the activation of apoptosis both in vivo and in vitro (28, 34). We determined caspase activity in BAD KD A549 cells infected with NY55 at an MOI of 1. Caspase activity was measured at 72 hpi using the Promega Caspase-Glo 3/7 kit. Non-transduced and stably transduced non-targeting shRNA cells were used as controls. Staurosporine-treated cells were included as positive controls for caspase-3/7 activity.

Infection of non-transduced and non-targeting shRNA cells resulted in an increase in caspase 3/7 activity by approximately 1.5-fold (P = 0.001; Fig. 7 A). We did not detect any significant difference in caspase activity induced by NY55 infection in BAD-deficient cells compared to uninfected mock (Fig. 7 A). Although there appeared to be a difference in caspase activity between the uninfected (mock) non-targeting shRNA control and BAD KD cells, this difference was not statistically significant (P = 0.234). Overall, this indicates that BAD is necessary for influenza virus activation of caspase 3/7 activity.

Influenza virus-induced cleavage of caspase-3 and caspase-7 is inhibited in BAD knockdown cells. The Caspase-Glo assay does not differentiate between the activities of caspase-3 and caspase-7. Given that caspase-3 activation is necessary for efficient influenza virus infection (37), we carried out immunoblotting for cleaved caspase-3 and caspase-7 protein...
products. Caspase activation requires proteolytic cleavage of zymogens into smaller, enzymatically active fragments (31). Cytoplasmic lysates were obtained from shRNA non-targeting control and BAD KD cells infected with NY55. Cleaved caspase-3 products were readily detected in the infected shRNA non-targeting control at 30 hpi, and most strongly visible at 72 hpi (Fig. 7 B, left column). However, caspase-3 cleavage was undetected at time points before 72 hpi and significantly reduced cleaved caspase-3 was detected in the infected BAD KD lane at 72 hpi (Fig. 7 B, right column).

We also observed caspase-7 cleavage in the infected non-targeting control, with significant cleavage occurring between 30 - 72 hpi (Fig. 8 A, left column). Similar to our observation with caspase-3, cleavage of caspase-7 was suppressed in BAD KD cells compared to the non-targeting control (Fig. 8 A, right column). Densitometric analysis of cleaved caspase-7 in the infected lanes showed on average a 5-fold reduction in caspase-7 cleavage compared to the shRNA non-targeting control cells (Fig. 8 B). These results suggest that influenza viruses need BAD in order to induce caspase activation.

Caspase-3 and -7 are effector caspases that cleave other proteins downstream of the apoptotic signaling pathway. One of these downstream substrates is PARP. We looked at PARP cleavage during influenza virus infection at specific time points late in the replication cycle in both the non-targeting control and BAD KD cells. Our results showed that influenza virus replication induces increasingly greater PARP cleavage from 14 hpi to 72 hpi (Fig. 8 A). However, cleaved PARP was only faintly detected in western blots of infected BAD KD cell lysates. Densitometric analysis showed more than a 10-fold reduction of PARP cleaved in the knockdown cells (Fig. 8 C). Interestingly, greater caspase-7 and PARP cleavage in the non-targeting control corresponded with an elevated expression of NS1 (Fig. 4 and Fig. 8). The
cleavage of both caspase-7 and PARP appeared most elevated at 30 hpi, suggesting this to be a critical period for apoptosis induction and viral replication. Further studies will provide a greater understanding of the critical molecular changes that may occur around this time period. Similar to our observation earlier, NS1 expression is reduced in BAD KD cells. In all, our data suggests that influenza viruses require the presence of BAD to efficiently induce the intrinsic apoptotic signaling pathway.

Discussion

Influenza virus induces cell death through activation of the apoptotic pathway, a process regarded as a major contributor to influenza virus pathogenesis that results in extensive lung tissue damage (3, 22). Blockage of the cell death pathway also leads to a significant decline in virus production (26). We report here that influenza virus-induced apoptosis requires BAD.

Our results showed that influenza viruses failed to induce cytopathology and cell death in BAD-deficient cells. Similar observations were reported for West Nile Virus (WNV) infection, where inhibition of apoptosis prevents WNV induction of cell death (14). Infection of BAD KD cells resulted in significant reduction in virus yield for all three virus strains we looked at (H3N2, H1N1, pandemic H1N1), and both early and late viral protein production were dramatically reduced. There is the possibility that the observed low viral protein production was a result of low progeny yield from initial infections, and therefore reduced viral spread and infection of other cells in the vicinity. However, a general reduction in overall viral protein produced during infection was also observed in Bcl-2 overexpressed cells (30). Thus, it is highly possible that BAD-deficient cells may fail to properly inhibit Bcl-2 activity.
BAD’s ability to associate and inhibit anti-apoptotic factors is regulated by phosphorylation at three residues: S112, S136, and S155 (6). A tiered phosphorylation and model of BAD inactivation has been proposed based on evidence that showed phosphorylation at S155 requires priming phosphorylation on S136 and S112 residues (6, 8). Sites S136, and especially S155, are reported to be more potent sites required for inactivating BAD activity (8, 24). Moreover, complete BAD inactivation requires phosphorylation of at least two serine residues (8). We determined that influenza viruses induce BAD phosphorylation of S112 and S136 sequentially from mid to late viral replication cycle. S112 phosphorylation occurred before S136, and both phosphorylation process gradually tapered off after 48 hpi. A study has shown that viral NS1 protein interacts with Akt, which results in enhanced Akt activity (25). S136 phosphorylation occurs via Akt in the PI3K signalling pathway (7, 10). We noticed an increase in NS1 protein production at 18 hpi to 20 hpi, whereas phosphorylation at S136 occurred from 20 hpi onward. This suggests that NS1 interaction with Akt may lead to the phosphorylation of BAD at residue S136. More studies are required to identify other cellular and/or viral factors that lead to the phosphorylation of BAD, and how these temporal interactions regulate BAD activity and modulate apoptosis during influenza virus replication.

Interestingly, we also noticed the appearance of a truncated, or cleaved, form of BAD starting at 48 hpi, with appearance of distinct cleavage at 72 hpi. BAD is cleaved by a number of caspases including caspase-3 and caspase-7 (5). The truncated form of BAD is reported to be a more potent inducer of cytochrome c release and apoptosis than the full-length form due to its higher affinity for Bcl-xL and the mitochondria membrane (5, 33). The N-terminally truncated BAD is poorly phosphorylated and has reduced affinity for 14-3-3, a cellular factor that inhibits BAD through direct association (8). It is proposed that caspase cleavage of BAD initiates a
mitochondrial amplification loop during apoptosis, similar to what has been observed with BID cleavage (19). It is possible that influenza viruses use BAD to moderate the degree of activation of the apoptotic pathway to enhance its replication but actively delays the complete onset of apoptosis that will lead to irreversible cell death. Thus, the virus moderates BAD activity by briefly inducing BAD phosphorylation and delays the appearance of the potent truncated form. Eventually, the death signaling factors will overwhelm the virus’ control and cell death will occur.

In the absence of BAD, we observed a suppression of cytochrome c release from the mitochondria, which is an early process of the intrinsic apoptotic cascade. Cytochrome c release will lead to the eventual downstream cleavage and activation of caspase-3 and caspase-7. Although the specific role of caspase-7 during influenza replication remains uncertain, it has been shown that caspase-3 cleavage is essential for influenza virus propagation (37). We show here that in BAD deficient cells, virus-induced cleavage of both caspase-3 and caspase-7 was inhibited. It is likely that the absence of BAD permitted the anti-apoptotic activity of Bcl-2 and Bcl-xL that resulted in blockage of cytochrome c release, and thus suppresses activation of downstream apoptotic factors such as the caspases. A reduction in caspase-3 activity would be a contributing factor to the inhibition of viral replication discussed above. The failure to activate caspase-3 in BAD KD cells was further supported by a reduction in PARP cleavage, which is a substrate of caspase-3 (16). Caspase-7 was considered to be functionally redundant with caspase-3 but recent studies have suggested that caspase-7 may be involved in the inflammatory response as well as apoptosis (17). Further studies are needed to clarify caspase-7’s role during influenza virus replication. Nevertheless, our data showed that caspase-7 is activated upon viral replication and suggests a potentially significant role this protease may play during influenza infection.
We have shown here that BAD is an important cellular factor required for influenza virus-induction of the apoptotic signaling cascade that is essential for efficient viral replication. A deficiency in BAD resulted in significant reduction in viral yield and suppress activation of the intrinsic apoptosis pathway. Our data suggest that BAD supports influenza virus replication through its innate role as a potent regulator of the mitochondria-dependent apoptotic pathway and as an antagonist of anti-apoptotic factors such as Bcl-2 and Bcl-xL. Our study presents evidence to further emphasize the importance of understanding the intricate relationship apoptosis has in promoting influenza virus propagation, and how its regulation may be a key to controlling influenza virus infection and prevention of host tissue damage.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.
References


Figure Legends

Figure 1. Influenza virus-induced cytopathology and cell death is inhibited in BAD knockdown cells. Cytopathic effect in infected (A) BAD-shRNA or (B) BAD-siRNA knockdown A549 cells that were infected with NY55, PR8, or SOIV. At 72 hpi, cytopathic effect of cells were examined with a Nikon Eclipse TE2000-S inverted microscope and images obtained with a Canon PowerShot A700 digital camera. Shown are images representative of 3 independent replicates. Cell viability at 72 h after influenza virus infection was determined for shRNA-treated cells by (C) WST-1 assay and by (D) trypan blue exclusion assay. A total of 200 cells were counted and the percentage of trypan blue-excluding (viable) cells was determined. NSi is nontargeting shRNA or siRNA control. Shown is the mean from 3 independent replicates with error bars representing standard deviation (* P < 0.001).

Figure 2. Efficiency of BAD knockdown in A549 cells. BAD transcripts were determined by real time PCR for (A) stably transduced shRNA cells (P < 0.001) or (B) siRNA-transfected cells (P = 0.002) and non-targeting (NSi) shRNA or siRNA control cells, respectively. Ct values were normalized to 18S rRNA control and compared to non-targeting (NSi) control. Effect of BAD knockdown on cell viability was assessed by WST-1 for (E) stably transduced shRNA cells and (F) siRNA-treated cells. Both cell viability and BAD mRNA quantification was determined 2 weeks after stably transduced cells were produced and 48 h after transfection with siRNA. NSi is nontargeting shRNA or siRNA control. Shown is the mean from duplicate runs with error bars representing standard deviation.
Figure 3. Inhibition of influenza virus replication in BAD-knockdown cells. (A) NY55 virus growth curve in shRNA-knockdown cells. (B) NY55 virus yield was determined at 72 hpi in shRNA-knockdown cells (P = 0.011). (C) NY55 virus yield in siRNA-knockdown cells (P < 0.001). (D) Virus yield of NY55, PR8, and SOIV replication at 72 hpi in shRNA-knockdown (P < 0.001) and (E) siRNA-knockdown A549 cells (P < 0.001). NSi is nontargeting shRNA or siRNA control. Error bars represent standard deviation from 3 independent experiments.

Figure 4. Influenza virus protein is reduced in BAD knockdown A549 cells. (A) Cells were infected with NY55 at MOI 1 and whole cell lysate was obtained at the indicated times. Western blot was probed with α-NS1, α-NP, and α-HA antibodies. (B) to (D) Densitometric quantitation of bands in infected lanes were done with Alpha Innotech FluorChem® Q Imaging System and normalized to β-actin. NSi is non-targeting shRNA control. Mock is uninfected control.

Figure 5. Influenza virus infection induces BAD phosphorylation and cleavage. A549 cells were infected with NY55 at MOI 3, harvested at indicated time points post-infection, and protein samples probed for phosphorylated BAD at residues S112 and S136, total BAD, and viral NS1 protein. Mock is uninfected.

Figure 6. Deficiency in BAD inhibits virus-induced cytochrome c release. Cytochrome c release was determined in NY55 infected cells at MOI 3. Cytosolic and mitochondrial fractions were obtained and blotted for cytochrome c at specific time points post-infection. Non-targeting represents non-targeting shRNA control. Mock is uninfected control.
Figure 7. BAD is required for influenza virus induced caspase-3 and caspase-7 activity. (A) Measurement of caspase-3/7 activity using Caspase-Glo assay at 72 hpi in A549 cells infected with NY55 at MOI 1, or at 24 h post-treatment of cells with 1 μM staurosporine. Shown is the mean from 3 independent replicates with error bars representing standard deviation (* P < 0.001). (B) Caspase-3 cleavage was assessed via Western blot for the small, cleaved fragment of caspase-3. Non-targeting control (NSi) and BAD-shRNA KD A549 cells were infected with NY55 at MOI 3t and cytoplasmic lysate was obtained at 72 hpi. NSi is non-targeting shRNA control.

Figure 8. Cleavage of caspase-7 and PARP is inhibited in influenza virus-infected BAD knockdown cells. (A) Non-targeting shRNA control (NSi) and BAD knockdown cells were infected with NY55 at MOI 3 and cells were harvested at specific times points. Whole cell lysates were subjected to Western blotting with antibodies to cleaved caspase-7, cleaved PARP, and viral NS1 proteins. (B) and (C) Densitometric quantitation of bands in infected lanes were done with Alpha Innotech FluorChem® Q Imaging System and normalized to β-actin. NSi is non-targeting shRNA control. Mock is uninfected control.
Table 1. shRNA and siRNA sequences to BAD mRNA.

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Table 2. Primers for real-time PCR of RNA transcripts.

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<th>Target Gene</th>
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| 18S rRNA*   | Fwd: TGAGAAACGGCTACCACATC  
              Rev: TTACGCGCCTCGAAAGAGT |
| BAD         | Fwd: ACCCGGCAGACAGATGAG  
              Rev: CTTTCTCTCACCAGTAGC |

* refer to (11)