Influenza Induces Endoplasmic Reticulum Stress, Caspase-12–Dependent Apoptosis, and c-Jun N-Terminal Kinase–Mediated Transforming Growth Factor- β Release in Lung Epithelial Cells

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Influenza A virus (IAV) infection is known to induce endoplasmic reticulum (ER) stress, Fas-dependent apoptosis, and TGF- β production in a variety of cells. However, the relationship between these events in murine primary tracheal epithelial cells (MTECs), which are considered one of the primary sites of IAV infection and replication, is unclear. We show that IAV infection induced ER stress marker activating transcription factor-6 and endoplasmic reticulum protein 57-kD (Erp57), but not C/EBP homologous protein (CHOP). In contrast, the ER stress inducer thapsigargin (THP) increased CHOP. IAV infection activated caspases and apoptosis, independently of Fas and caspase-8, in MTECs. Instead, apoptosis was mediated by caspase-12. A decrease in Erp57 attenuated the IAV burden and decreased caspase-12 activation and apoptosis in epithelial cells. TGF- β production was enhanced in IAV-infected MTECs, compared with THP or staurosporine. IAV infection caused the activation of c-Jun N-terminal kinase (JNK). Furthermore, IAV-induced TGF- β production required the presence of JNK1, a finding that suggests a role for JNK1 in IAV-induced epithelial injury and subsequent TGF- β production. These novel findings suggest a potential mechanistic role for a distinct ER stress response induced by IAV, and a profibrogenic/repair response in contrast to other pharmacological inducers of ER stress. These responses may also have a potential role in acute lung injury, fibroproliferative acute respiratory distress syndrome, and the recently identified H1N1 influenza-induced exacerbations of chronic obstructive pulmonary disease (Wedzicha JA. *Proc Am Thorac Soc* 2004;1:115–120) and idiopathic pulmonary fibrosis (Umeda Y, *et al. Int Med* 2010;49:2333–2336).

Keywords: ER stress; influenza A virus; ATF6; Erp57; TGF- β

Influenza A virus (IAV) is a highly infective cytolytic virus affecting humans, and annually causes approximately 250,000 deaths worldwide (1, 2). The genome of IAV is made up of segmented, single-stranded negative-sense RNA (3). IAV infection and

CLINICAL RELEVANCE

Influenza virus, apart from causing severe respiratory illness, has also been associated with exacerbations of chronic lung disease. Our study demonstrates that influenza infection induces endoplasmic reticulum stress, caspase-12–mediated apoptosis, and c-Jun N-terminal kinase–mediated transforming growth factor- β (TGF- β) production in primary lung epithelial cells, which are known to be the primary site of influenza infection and replication. Collectively, the results provide a mechanistic link between influenza-induced epithelial injury and TGF- β production, and highlight potential therapeutic targets during exacerbations of chronic lung disease.

replication in cells leads to the activation of double-stranded RNA (dsRNA) activated protein kinase (PKR) and the subsequent phosphorylation of elongation factor eIF2 α , resulting in the down-regulation of protein synthesis (4, 5). Influenza viral proteins use cellular chaperone proteins such as endoplasmic reticulum protein 57-kD (Erp57), which belongs to the family of protein disulfide isomerases, for the folding and maturation of proteins (6). An increased production of proteins results in the unfolded protein response (UPR) and the activation of endoplasmic reticulum (ER) stress–mediated transcription factors, such as activating transcription factor-6 (ATF6) and X-box binding protein-1. These transcription factors further increase chaperone protein and antioxidant enzyme expression to cope with the stress exerted by increased protein synthesis. When the UPR becomes irresolvable, the cell activates C/EBP homologous protein (CHOP), an inducer of cell death (7). Although these are the general mechanisms of ER stress and UPR, the activation of ATF6, Erp57, and CHOP has not been well documented during IAV (referred to henceforth as influenza virus) infection.

Influenza virus–induced apoptosis is reportedly mediated by both Fas-dependent (as analyzed by expression profiling in influenza-infected A549 cells) (8) and Fas-independent mechanisms. The Fas-dependent mechanism, upon influenza virus infection, requires an up-regulation of Fas ligand (FasL), which in turn induces the Fas apoptotic cascade. FasL-independent cell death is mediated by viral RNA replication and PKR kinase by indirectly promoting Fas-associated death domain (FADD)–caspase-8 interactions, resulting in apoptosis (8). Another known pathway of apoptosis induced by influenza virus occurs through the autocrine effects of transforming growth factor- β (TGF- β) (9).

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Figure 1. Influenza virus infection induces endoplasmic reticulum (ER) stress and caspase activity. Wild-type (WT) primary murine tracheal epithelial cells (MTECs) were infected with influenza virus for the indicated times. (A) ER stress was assessed by Western blotting for ER stress-induced activating transcription factor-6 (ATF6; 50 kD), chaperone endoplasmic reticulum protein 57-kD (ERp57), and actin as a loading control. (B) Caspase activity was measured by a luminescence assay for caspase-8, caspase-9, and caspase-3. * $P < 0.05$ (ANOVA), compared with mock control. (C) Assessment of influenza virus infection by quantitative RT-PCR analysis of the viral polymerase (PA) gene. Ultraviolet light (UV)-irradiated, replication-deficient influenza virus (labeled as "Mock") was used as control. (D) Assessment of influenza virus infection in MTECs grown at an air-liquid interface (ALI), by quantitative RT-PCR analysis of the viral PA gene. (E) Western blot analysis of ER stress markers induced by influenza virus in MTECs grown at an ALI. (F) Assessment of caspase-3 activation in MTECs grown at an ALI upon infection with influenza virus. UV-irradiated, replication deficient influenza virus (labeled as "Mock") was used as control. hr, hours; IAV, influenza A virus; RLU, relative luminescence units.

Influenza virus infection in humans was reported to cause acute respiratory distress syndrome (ARDS) (10). Moreover, approximately 6–8% of patients with chronic H1N1 influenza infection also developed diffuse alveolar damage and pulmonary fibrosis (2, 11). Although TGF- β and FasL were shown to be involved in ARDS-induced tissue remodeling and fibrosis (12, 13), whether these two potent fibrotic mediators are involved in influenza virus-induced fibroproliferative ARDS remains unclear.

Substantial *in vitro* information on influenza virus-induced ER stress and apoptosis has been obtained using A549 cells (a human lung carcinoma cell line) (8), the Madin Darby canine kidney (MDCK) cell line (9), murine embryonic fibroblasts, or murine primary lung fibroblasts (14, 15). Although these studies provide valuable insights into the mechanisms of ER stress, inflammatory cytokine production, and apoptosis, the pathway of influenza virus-induced ER stress and apoptosis in primary murine tracheal epithelial cells (MTECs), one of the primary targets of influenza virus infection and replication (2), remains

unclear. Therefore, this study was designed to evaluate whether influenza virus infection leads to a specific ER stress response and Fas-dependent apoptosis, and additionally whether these events coincide with the production of the profibrogenic mediator, TGF- β . We further sought to compare the influenza virus-induced ER stress response with that induced by pharmacological ER stressors.

In this study, we demonstrate for the first time, to the best of our knowledge, that the influenza virus infection of MTECs leads to an increase in the ER stress-triggered transcription factor ATF6, and the ER chaperone ERp57. Fas and caspase-8 were dispensable in influenza virus-induced ER stress and apoptosis. In contrast, influenza virus-induced apoptosis and replication were mediated by caspase-12. Moreover, TGF- β was specifically produced by influenza virus-infected MTECs in a c-Jun N-terminal kinase (JNK)-1-dependent manner. These results suggest a putative role for ER stress, caspase-12, and JNK-1 in influenza virus-induced apoptosis and the production of

Figure 2. Fas and caspase-8 are dispensable for influenza virus-induced apoptosis. MTECs derived from WT and Fas-deficient *lpr* mice were infected with influenza virus. (A) ER stress was assessed by Western blotting for ER stress-induced transcription factor ATF6 (50 kD), chaperone ERp57, and actin as a loading control. (B) Caspase activity was measured by a luminescence assay for caspase-8, caspase-9, and caspase-3. * $P < 0.05$ (ANOVA), compared with mock control. (C) Cell death was measured by dead cell protease activity in the culture supernatants. * $P < 0.05$ (ANOVA), compared with mock control. (D) WT MTECs were treated with cell-permeable caspase-8 inhibitor (IETD-FMK) and infected with IAV for the indicated times. Western blots reveal ATF6 (50 kD) and ERp57. (E) Assessment of caspase-8, caspase-9, and caspase-3 activities by luminescence assay. * $P < 0.05$ (ANOVA), compared with mock control. (F) Cell death was measured in terms of dead cell protease activity in the culture supernatants. * $P < 0.05$ (ANOVA), compared with UV-inactivated mock control. CHOP, C/EBP homologous protein; RFU, relative fluorescence units.

fibrosis mediator TGF- β in MTECs. These findings demonstrate that primary tracheal epithelial cells infected with influenza virus follow mechanistically distinct pathways to induce apoptosis and the production of TGF- β . Some of these data were presented previously in abstract form.

MATERIALS AND METHODS

Cells and Treatments

Primary MTECs were isolated and cultured from C57BL/6 mice, Fas-deficient *lpr* mice, and *Jnk1*^{-/-} mice in the same genetic background as described previously (16). To differentiate cells, MTECs were plated onto trans-wells and treated with retinoic acid for 10 days in an air-liquid interface (ALI), and differentiation was confirmed by dot blots for mucin (MUC5AC), as described previously (16). Type II epithelial cells (C10s) were cultured as described by Velden and colleagues (17). Cells were plated at 2×10^6 cells/dish or 5×10^5 cells per trans-well, and when they were 80% confluent or at $1,000 \Omega \times \text{cm}^2$ (MTECs in ALI), they were infected with mouse-adapted H1N1 influenza A virus Puerto Rico 8/34 (PR8) at 2 Egg infectious units/cell in a Dulbecco's modified Eagle's medium (DMEM)F12 growth factor-free medium (for submerged cultures) for the indicated times. Ultraviolet light (UV)-irradiated virus that was replication-deficient (mock) was used as a control. For ALI cultures, cells were infected in the top well with 50 μ l of growth factor-free medium, and the medium was

removed after 6 hours of infection. Thapsigargin (Sigma, St. Louis, MO) was used at a concentration of 50 nM, and DMSO was used as control. Staurosporine (Sigma) was used at 1 μ M for the indicated times. For the inhibition of caspase-8 or caspase-12, MTECs were preincubated with 10 μ M of cell-permeable caspase-8 inhibitor Z-IETD-FMK (R&D, Minneapolis, MN) or 30 μ M of cell-permeable caspase-12 inhibitor Z-ATAD-FMK (MBL, Woburn, MA) for 2 hours. During infection, and every 24 hours, cultures were replenished with the inhibitors. All treatments were performed in a growth factor-free medium. For details on quantitative RT-PCR and Western blots, see the online supplement.

Caspase Assay

Cells were lysed at the indicated times. Caspase activity was measured using a Caspase-Glo assay (Promega, Madison, WI). Values were expressed as relative luminescence units.

Measurement of Cell Viability

Apoptosis in influenza virus-infected MTECs or C10 cells was measured using the Apo Tox-Glo assay (Promega) dead cell protease substrate, with some modifications. Briefly, the cell-culture supernatants were centrifuged at 5,000 rpm for 5 minutes to clear the cells and cell debris. Two hundred microliters of supernatant were mixed with the dead cell protease substrate bis-AAF-R110 in a 96-well plate and

Figure 3. Caspase-12 mediates influenza-induced apoptosis in a Fas-independent manner. (A) WT or Fas-deficient (*lpr* genotype) MTECs were incubated with the cell-permeable caspase-12 inhibitor (ATAD) or DMSO as a vehicle control. Cells were then infected with IAV or UV-irradiated virus (Mock). At the indicated times, influenza virus infection was measured by quantitative RT-PCR analysis of viral PA mRNA. * $P < 0.05$, compared with UV-inactivated mock control. (B) ER stress, the presence of Fas, total caspase-12 (T-C12), and activated caspase-12 (A-C12) were evaluated by Western blotting for ATF6 (50 kD), ERp57, Fas, and 45-kD and 35-kD total and active caspase-12 fragments. Actin was used as a loading control. (C) Caspase-3 activity was measured by a luminescence assay. * $P < 0.05$, compared with 48-hour IAV-infected, DMSO-treated samples. (D) Cell death was measured by dead cell protease activity in the supernatants. * $P < 0.05$, compared with 48-hour IAV-infected, DMSO-treated samples.

incubated for 30 minutes at 37°C. After 30 minutes, protease activity was measured by fluorescence (485_{EX}/520_{EM}). Values obtained were then subtracted from blank (media and substrate alone) values, to be expressed as relative fluorescence units. Apoptosis from thapsigargin-treated cells were measured using a (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described elsewhere (18). The values in graphs represent three experiments performed in triplicate.

ELISA

Supernatants were collected from treated and control plates at the indicated times. ELISAs for TGF- β and IL-6 were performed according to the manufacturer's protocol (R&D).

Statistical Analysis

Results were analyzed using one-way ANOVA, with the Tukey honestly significant difference test for multiple comparisons. Results at $P < 0.05$ or less were considered statistically significant. All values are expressed as mean values $\pm$ SEM. All graphs represent combined values of two to three experiments performed in triplicate (i.e., 6–9 plates).

RESULTS

IAV Infection Induces ER Stress and Caspase Activation

MTECs derived from wild-type (WT) mice were infected with influenza virus, and cell lysates were analyzed for ER stress markers. Twenty-four hours after infection, we found an increase in ATF6 (50 kD), which was sustained up to 48 hours after infection (Figure 1A). The ER chaperone, ERp57, was also increased during the same time points, indicating influenza virus-induced ER stress in infected MTECs. MTECs that were treated with UV-irradiated, replication-deficient virus (mock)

did not show any increase in ER stress markers, even after 48 hours of incubation, suggesting that viral replication and protein production are required to induce ER stress. Next, we analyzed whether ER stress induction was associated with the activation of caspases. By 24 hours, MTECs infected with influenza virus showed increased activity of caspase-8, caspase-9, and the apoptosis effector caspase-3, which was sustained until 48 hours after infection, and was correlated with the presence of virus (Figure 1C). The use of mock virus-treated cells did not result in an increase in caspase activity, or the presence of virus, even after 48 hours (Figures 1B and 1C). These results indicate that influenza virus infection induces ER stress and the activation of all three caspases involved in apoptosis. Furthermore, to test whether differentiated epithelial cells also respond in a similar manner, we cultured the MTECs in an ALI (16) before infection with influenza virus. The results in Figure 1D demonstrate that infection with influenza virus occurred under ALI conditions, based on increases in concentrations of polymerase (PA) mRNA of the influenza virus according to quantitative RT-PCR. The results also demonstrate that influenza virus activated ER stress markers (Figure 1E) and caspase-3 (Figure 1F) in ALI cultures (16). Because both culture conditions showed similar results, we performed subsequent experiments in submerged cultures.

Fas and Caspase-8 Are Not Required for Apoptosis during IAV Infection

To analyze the requirement for the death receptor Fas in influenza virus-triggered ER stress-associated apoptosis, we infected MTECs derived from WT and Fas-deficient *lpr* mice with influenza virus, and then analyzed the cell lysates for ER

Figure 4. ERp57 is required for influenza virus propagation. (A) C10 lung epithelial cells were transfected with control (ctr) small interfering (si)RNA (Ctrsi) or ERp57 siRNA before infection with influenza virus or UV-inactivated mock virus. ER stress, the presence of ERp57, total caspase-12 (T-C12), and activated caspase-12 (A-C12) were evaluated by Western blotting for ATF6 (50 kD), ERp57, and 35-kD active caspase-12 fragments. Actin was used as loading control. (B) Influenza virus infection was measured by quantitative RT-PCR analysis of viral PA mRNA. * $P < 0.05$, compared with 48-hour and 72-hour influenza virus-infected ctr siRNA-transfected cells. (C) Caspase-3 activity was measured by a luminescence assay. * $P < 0.05$, compared with 48-hour and 72-hour influenza-infected ctr siRNA-transfected cells. (D) Cell death was measured by dead cell protease activity in the supernatants. * $P < 0.05$, compared with 48-hour and 72-hour influenza-infected ctr siRNA samples.

stress markers via Western blot analysis. In the cells derived from both WT and *lpr* mice, ATF6 (50 kD) and ERp57 were increased at 24 hours, and this increase was sustained until 48 hours after infection (Figure 2A). We could not detect any expression of the ER stress-dependent death inducer CHOP, indicating that CHOP may not be involved in influenza virus-mediated ER stress and apoptosis in these cells. Next, we analyzed caspase activation in infected cells. WT and *lpr* cells also showed similar levels of activation of caspase-8, caspase-9, and caspase-3 after influenza virus infection (Figure 2B), indicating that Fas is not required for the ER stress-induced activation of caspases in primary MTECs. In addition, we found that influenza virus induced comparable cell death in WT and *lpr* cell types (Figure 2C). We then tested directly whether caspase-8 is required for the activation of caspase-9 and caspase-3 by blocking caspase-8 activation, using the inhibitor Z-IETD-FMK. Western blot analysis of cell lysates for ER stress markers demonstrated that cells treated with IAV $\pm$ Z-IETD-FMK showed increases in ATF6 and ERp57 by 24 hours, which were sustained until 48 hours after infection (Figure 2D). Furthermore, MTECs did not show any inhibition of caspase-9 and caspase-3 (Figure 2E), although as expected, caspase-8 was inhibited in MTECs treated with Z-IETD-FMK. Lastly, we observed similar increases in cell death in influenza virus-infected, DMSO-treated and Z-IETD-FMK-treated MTECs (Figure 2F). These results suggest a lack of involvement for caspase-8 in the activation of caspase-9 and caspase-3 and cell death in response to influenza virus. These results indicate that both Fas and caspase-8 are dispensable in influenza virus-induced apoptosis.

Thapsigargin Induces CHOP Instead of ATF6 and ERp57, and Does Not Require Fas to Induce Apoptosis

Thapsigargin (THP) is widely used as an ER stress inducer, and is known to increase CHOP in a time-dependent and concentration-dependent manner in various cell types (19). Because no activation of CHOP occurred with influenza virus infection, we tested whether MTECs could activate CHOP in response to THP. We treated WT and *lpr* MTECs with a dose of THP (50 nM) that was shown to induce ATF6 and CHOP (19). In contrast to the results with influenza virus, no induction of ATF6 or ERp57 was detected in THP-treated WT or *lpr* cells compared with control samples. Instead, THP-exposed cells expressed CHOP (Figure E1A), indicating that ER stress had been induced. THP also activated

caspases (Figure E1B) and cell death (Figure E1C) in WT and *lpr* cells to a similar extent, again suggesting a lack of involvement for Fas in the induction of caspases and cell death after THP-induced ER stress. Collectively, these findings indicate that both influenza virus and THP-induced ER stress activate caspases independently of Fas. However, the patterns of ER stress were observed to be stimulus-specific.

Influenza Virus-Induced Apoptosis Is Mediated by Caspase-12

Caspase-12 is known to mediate ER stress-induced apoptosis in cells (20). Therefore, to elucidate the mechanism leading to apoptosis, we tested whether influenza virus-infected MTECs undergo caspase-12-mediated apoptosis. WT and *lpr* MTECs were infected with influenza virus and treated with DMSO or the caspase-12 inhibitor Z-ATAD-FMK. Semiquantitative RT-PCR analysis of the viral PA gene showed similar influenza virus PA mRNA in influenza virus-infected DMSO or Z-ATAD-FMK-treated cells (Figure 3A). Furthermore, influenza virus-infected DMSO or ATAD-FMK-treated cells activated ATF6 and ERp57 (Figure 3B) in a similar manner. As expected, no caspase-12 activation was observed in ATAD-FMK-treated cells after infection with influenza virus (Figure 3B), and ATAD-FMK largely prevented the influenza virus-induced activation of caspase-3 (Figure 3C) and cell death (Figure 3D). These results strongly suggest a caspase-12-dependent mechanism of cell death in influenza virus-infected MTECs.

Knockdown of ERp57 Decreases Virus Burden and Attenuates Cell Death

The ER chaperone ERp57 is known to be involved in folding the hemagglutinin (HA) protein of influenza virus (6). Because we demonstrated that infection with influenza virus increased ERp57 protein content, we speculated that decreasing ERp57 would affect HA folding and progeny virion assembly, and subsequently protect epithelial cells from influenza virus-mediated apoptosis. To test this, we decreased the protein content using ERp57 small interfering (si)RNA and subsequently infected lung epithelial cells with influenza virus. The results in Figure 4A show that we could successfully knock down ERp57 in these cells, and this knockdown not only attenuated the increase in ERp57, but also reduced ATF6. Importantly, the knockdown of ERp57 also prevented the IAV-induced activation of caspase-12

Figure 5. Influenza virus induces IL-6 and transforming growth factor- β (TGF- β). (A) Supernatants from MTECs treated with IAV, thapsigargin (THP), and staurosporine (STS) were collected, and ELISA was performed for IL-6. * $P < 0.05$, compared with UV-inactivated mock or DMSO controls. (B) ELISA for TGF- β from the culture supernatants. * $P < 0.05$, compared with UV-inactivated mock control. # $P < 0.05$, compared with THP 48-hour and STS 48-hour samples, respectively. (C) Assessment of TGF- β mRNA in IAV-infected MTECs grown at an air-liquid interface by quantitative RT-PCR analysis. * $P < 0.001$.

(Figure 4A). The quantification of viral *PA* mRNA in control siRNA and ERp57 siRNA-treated cells demonstrated that cells lacking ERp57 had significantly reduced virus *PA*, suggesting a lack of propagation of the virus (Figure 4B). Furthermore, ERp57 siRNA-treated cells also showed a significant reduction in caspase-3 activity and cell death (Figures 4C and 4D). Collectively, these results demonstrate that ERp57 is required for the replication of influenza virus and for influenza virus-induced increases in ATF6, the activation of caspase-3 and caspase-12, and cell death.

Influenza Virus Infection Induces TGF- β Production in MTECs

As previously mentioned, airway epithelial cells, the primary site of influenza virus infection, are known to participate in the release of inflammatory mediators and cytokines (21–24). To

investigate whether the distinct ER stress response observed in this study is associated with the production of inflammatory and profibrotic cytokines, we analyzed IL-6 production in culture supernatants of MTECs infected with influenza virus or treated with THP or staurosporine, which is known to induce caspase-3-mediated cell death. The results in Figure 5A demonstrate that in response to influenza virus, THP or staurosporine concentrations of IL-6 were increased to differing extents. Earlier reports indicated that influenza virus induces TGF- β production from MDCK cells, and that active TGF- β was increased in the bronchoalveolar lavage fluid and lung homogenates of mice infected with influenza virus (9, 25). Thus, we examined whether the influenza virus infection of MTECs induced TGF- β release. The results in Figure 5B demonstrate that TGF- β production was increased in the supernatants of MTECs infected with influenza virus, but not in response to THP or staurosporine. Furthermore, our analysis in ALI cultures showed increased concentrations of TGF- β mRNA (Figure 5C), confirming that influenza infection in ALI cultures results in the production of TGF- β .

Ablation of JNK-1 Attenuates Influenza Virus-Induced TGF- β Production but Not Apoptosis

Numerous studies showed that ER stress can activate JNK (7). Recent reports also suggest that ER stress can increase the phosphorylation of JNK and subsequent proinflammatory signaling, and JNK is known to regulate TGF- β transcription (26, 27). In addition, the activation of JNK is known to induce apoptosis in various cell types (18, 28). However, no studies have mechanistically linked influenza virus-induced ER stress, activated JNK in apoptosis, and TGF- β production. Therefore, to elucidate that the phosphorylation of JNK plays a role in influenza virus-induced apoptosis and TGF- β production, we infected WT and *Jnk1*^{−/−} primary tracheal epithelial cells with influenza virus for different lengths of time. Analysis of influenza virus polymerase expression showed that influenza virus infected both cell types efficiently (Figure 6A). Western blots for ATF6 and ERp57 showed that these two ER stress markers were activated in a similar manner. Analysis using a phosphorylated (P) JNK antibody showed that influenza virus-infected WT cells activated JNK, a result that was not observed in *Jnk1*^{−/−} MTECs (Figure 6B), suggesting that influenza virus infection predominantly activates JNK-1. WT and *Jnk1*^{−/−} cells showed a similar extent of caspase-3 activation and apoptosis in response to infection with influenza virus (Figures 6C and 6D). However, after infection with influenza virus, *Jnk1*^{−/−} cells produced significantly lower amounts of IL-6 (Figure 6E) compared with WT or *lpr* MTECs. Intriguingly, TGF- β 1 production was entirely abolished at the level of protein (Figure 6F) as well as mRNA in *Jnk1*^{−/−} cells (Figure 6G), demonstrating that JNK-1 phosphorylation plays a major role in the influenza virus-induced production of TGF- β 1. Lastly, to confirm the direct involvement of influenza virus infection-induced ER stress activation, JNK phosphorylation, and TGF- β production, we analyzed JNK phosphorylation and TGF- β 1 mRNA expression in ERp57 siRNA-transfected C10 cells, which showed an attenuation of viral propagation and ATF6 activation (Figure 4). As demonstrated in Figure 6B, the influenza virus-induced phosphorylation of JNK in control siRNA-transfected cells. In contrast, no activation of JNK was detected in ERp57 siRNA-transfected cells (Figure 7A). ERp57 siRNA also significantly decreased influenza virus-mediated TGF- β 1 mRNA (Figure 7B). These results strongly suggest that influenza virus-triggered ER stress activates caspase-12 and JNK to induce apoptosis as well as TGF- β production in primary MTECs (Figure 7C).

Figure 6. c-Jun N-terminal kinase (JNK)-1 mediates influenza virus-induced TGF- β production. (A) WT, *Jnk1*^{-/-}, or *lpr* MTECs were infected with influenza virus or UV-inactivated mock virus for the indicated times. Influenza virus infection was measured by quantitative RT-PCR analysis of viral PA mRNA. **P* < 0.05, compared with mock control. (B) ER stress, phosphorylation of JNK-1 (P-JNK), and total JNK (T-JNK) were evaluated by Western blotting. Actin was used as loading control. (C) Caspase-3 activity was measured by a luminescence assay. **P* < 0.05, compared with UV-inactivated mock virus samples. (D) Cell death was measured by dead cell protease activity in the supernatants. **P* < 0.05, compared with mock infected samples. (E) Supernatants from WT, *lpr*, and *Jnk1*^{-/-} MTECs treated with influenza virus were collected, and ELISA was performed for IL-6. **P* < 0.05, compared with mock controls. [§]*P* < 0.05, compared with WT and *lpr* 48-hour influenza-infected samples. (F) ELISA for TGF- β . **P* < 0.05, compared with WT and *lpr* 48-hour infected samples. (G) TGF- β mRNA was measured by quantitative RT-PCR analysis. **P* < 0.001, compared with WT and *lpr* 48-hour influenza-infected samples.

DISCUSSION

The ER stress response is a mechanism to repair misfolded proteins during physiological stress. When the damage from stress becomes irreparable, the host initiates a programmed death cascade (7). Although many investigators have studied influenza virus-induced ER stress and apoptosis in an array of cell lines (14, 15, 29), this, to the best of our knowledge, is the first study showing an association of influenza virus-triggered ER stress with viral replication, caspase-12-dependent cell death, and TGF- β production in primary tracheal epithelial cells. Specifically, we demonstrated that the influenza virus-induced ER stress response activates ATF6 and increases ERp57, but not CHOP. ERp57 was shown to be involved in folding HA, a protein of influenza virus that is necessary for virion assembly and propagation. However, the increasing concentrations of ERp57 in response to the influenza virus infection of MTECs were not previously documented. We speculate that an increase in ERp57 would help influenza virus to assemble efficient virions and enhance infection. Our results also specifically demonstrate that influenza virus induces ERp57 but not the pharmacological ER stress inducer THP.

Previous studies showed that IAV induces Fas-mediated apoptosis in a variety of cells (6, 15). Other studies in fibroblasts suggested a PKR-mediated, dsRNA-dependent interaction between FADD and caspase-8, leading to apoptosis (21, 23, 30). A recent study also showed that ER stress increases apoptosis in peritoneal macrophages in a Fas-dependent manner (31). However, those results were not confirmed in the present study, which used primary lung epithelial cells, known to be the primary site of infection for influenza virus (32). Our study indicated that lung epithelial cells do not require Fas for apoptosis upon infection with influenza virus or THP. Further, our results showed that both Fas and caspase-8 are dispensable for cell death induced by influenza virus. Interestingly, the ER stress

mediators induced by influenza virus were distinctly different compared with those induced by THP, despite a similar caspase activation.

Caspase-12 is known to mediate ER stress-induced apoptosis. Cells deficient in caspase-12 are partly resistant to ER stress-induced apoptosis (20). In the normal physiological state, caspase-12 was shown to be bound by tumor necrosis factor receptor-associated factor-2 (TRAF2). During ER stress, TRAF2 is released from caspase-12, facilitating the activation of caspase-12, which in turn activates caspase-9 and subsequently activates caspase-3 to induce cell death (33, 34). In accordance with these findings, our study indicated that influenza virus infection in MTECs activates caspase-12, and that the inhibition of caspase-12 resulted in a reduced activation of caspase-3 and influenza virus-induced apoptosis. Furthermore, we speculate that in our experiments, the activation of caspase-3 may have been preceded by the activation of caspase-9. These results indicate that influenza virus-induced ER stress and the subsequent induction of caspase-12 mediates Fas/caspase-8-independent apoptosis in primary lung epithelial cells.

Influenza virus is well known to host protein synthesis and processing machinery such as the ER for efficient virion assembly (35). The host protein ERp57 is known to be involved in the folding of influenza HA *in vitro* (6). Our novel results show that influenza virus infection increases ERp57 in primary lung epithelial cells. Furthermore, a reduction in concentrations of ERp57 in Type II epithelial cells (C10) substantially decreased viral particle production and attenuated caspase-12 activation and apoptosis. The observed attenuation of caspase-12 activity may be attributable to a lack of viral particle production, resulting in a reduction in ER stress. Furthermore, this attenuation of caspase-12 may have contributed to the reduction in cleavage of caspase-9 and subsequent activation of caspase-3. We also observed that the influenza virus infection was less pronounced in C10 cells compared with MTECs. Nevertheless, C10 cells

Figure 7. Influenza virus-induced ER stress activates JNK and mediates TGF- β production. (A) C10 cells were transfected with control siRNA or ERp57 siRNA before infection with influenza or UV-inactivated mock virus for the indicated times. ER stress (ERp57), the phosphorylation of JNK (P-JNK), and total JNK (T-JNK) were evaluated by Western blotting. Actin was used as loading control. (B) TGF- β 1 mRNA was measured by quantitative RT-PCR analysis. $^{*}P < 0.001$, compared with influenza-infected 48-hour and 72-hour samples, respectively. (C) The proposed model shows influenza virus infection triggers ER stress, which subsequently activates caspase-12 and JNK, resulting in cell death and TGF- β production, respectively. This pathway could be inhibited at three key points: (1) the inhibition of ER stress response by ERp57 siRNA reduces viral burden and attenuates downstream events; (2) the inhibition of caspase-12 can rescue epithelial cells from influenza-triggered ER stress-mediated cell death; and (3) the inactivation of JNK can result in an attenuation of TGF- β production. ATAD-FMK, cell-permeable caspase-12 inhibitor.

activated both components of ER stress (i.e., cell death and TGF- β production), suggesting that these responses were intact in Type II epithelial cells. Collectively, these results indicate that ERp57 plays a role in viral particle assembly, and could be a potential therapeutic target to control influenza virus infection.

ER transmembrane protein inositol requiring enzyme 1 α (IRE1 α) plays a pivotal role in UPR-dependent pathways in the cell (34). The activation of IRE1 α by UPR results in the phosphorylation of JNK and mitochondria-dependent caspase activation (36). A recent report also showed that ER stress activated IRE1 α -induced JNK and proinflammatory cytokines in lung epithelial cells (26). Interplay is known to occur between JNK and TGF- β . Active TGF- β initiates a signaling cascade, leading to the activation of JNK and resulting in the activation of transcription factors and the up-regulation of proapoptotic

gene expression (21). Conversely, coculture studies show that apoptotic Jurkat cells activate JNK and induce TGF- β production in normal fibroblasts (27). Further, the influenza virus neuraminidase is known to activate TGF- β (9, 25). Our study adds a new link toward understanding the mechanism of regulation of profibrotic cytokine TGF- β . For the first time, to the best of our knowledge, we show that influenza virus-mediated ER stress activates JNK, and this activation of JNK further regulates profibrotic mediator TGF- β production. Investigations with ERp57 siRNA-treated and *Jnk1*^{-/-} epithelial cells demonstrated that influenza virus-induced TGF- β production was dependent on ERp57 and JNK in lung epithelial cells. Although our study does not show the direct link between IRE1 α and P-JNK, further research should reveal the involvement of IRE1 α in influenza virus-induced JNK activation and TGF- β production. Further, our results strongly indicate that the influenza virus-induced apoptosis of primary tracheal epithelial cells was not attributable to JNK1 or TGF- β , instead occurring in a caspase-12-dependent manner.

TGF- β is known to mediate pulmonary fibrosis in humans as well as in murine models. Patients infected with novel H1N1 or avian H5N1 influenza manifest ARDS, diffuse alveolar cell damage, and fibrosis during convalescence (15). Interestingly, a recent report suggests that reo virus (a dsRNA virus) mediates the development of ARDS and pulmonary fibrosis, and occurs independent of Fas signaling (37). Thus, further investigation should reveal whether influenza or RNA viruses generally trigger ATF6, ERp57, and subsequent apoptosis, and whether these proteins promote TGF- β production and fibrogenesis in an ER stress-dependent, but Fas-independent manner.

The proinflammatory cytokine IL-6 is known to be produced from IAV-infected cell lines. Th17 cell development requires TGF- β and IL-6/IL-21 (38). Interestingly, a recent report demonstrated that IL-17A produced by TH17 cells can mediate fibrosis in mice (39). Hence, further investigation should reveal whether TGF- β and IL-6 produced by epithelial cells would enhance the Th17 lineage and promote fibrosis in response to influenza virus.

In conclusion, for the first time, to the best of our knowledge, we have shown that influenza virus-induced ER stress activates ATF6 and ERp57, but not CHOP. This distinct activation of the ER stress response elicits multiple outcomes, including caspase-12-dependent apoptosis and JNK1-dependent TGF- β production in lung epithelial cells (Figure 7C). This pathway could be inhibited at several key points: (1) the inhibition of the ER stress response by ERp57 siRNA may reduce viral burden and attenuate downstream events, (2) the inhibition of caspase-12 can rescue epithelial cells from IAV-triggered ER stress-mediated cell death, and (3) the inactivation of JNK can result in the attenuation of TGF- β production. In the future, the development of specific inhibitors for these molecules may have therapeutic value in terms of influenza infection and disease severity.

Author disclosures are available with the text of this article at www.atsjournals.org.

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