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# Asthma and Influenza Virus Infection: Focusing on Cell Death and Stress Pathways in Influenza Virus Replication

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**ABSTRACT**

Asthma is one of the fastest growing syndromes in many countries and is adding a huge cost to the health care system. Increasing reports have linked airway infectious diseases to asthma. Influenza is one of the most serious airway infectious diseases and in recent years there have been some serious influenza virus pandemics which caused increased fatality in numerous different populations. Diverse host response pathways during virus infection have been identified, including different cell death and survival pathways. These pathways include 1) programmed cell death I (apoptosis), 2) programmed cell death II (autophagy), and 3) endoplasmic reticulum stress with subsequent unfolded protein response (UPR). There has been extensive research on the regulatory roles of these pathways during the influenza virus life cycle. These studies address the benefits of enhancing or inhibiting these pathways on viral replication. Here we review the most recent and significant knowledge in this area for possible benefits to clinicians and basic scientist researchers in different areas of the respiratory and virology sciences.

**Keywords:** Apoptosis, Autophagy; Endoplasmic Reticulum Stress; Unfolded Protein Response; Virus-induced autophagy; Virus-induced apoptosis

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## Importance of Influenza Virus infection in Asthma and Allergy

Asthma is the most common chronic disease among children and young adults and is a major public health problem that affects nearly 300 million

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people worldwide.[1] Asthma is a chronic inflammatory disorder of the airways characterized by reversible airway obstruction and hyperresponsiveness (AHR) associated with pulmonary inflammation, airway wall remodeling and mucus overproduction. These events are believed to be coordinated by eosinophils and basophils in combination with allergen-specific T helper type 2 ($T_H2$) cells.[2] More specifically, asthma is thought to arise from an imbalance in T helper type 1 ($T_H1$)-$T_H2$ immune regulation, resulting in increased $T_H2$ cytokines (IL-4, IL-5 and IL-13) as well as increased immunoglobulin E (IgE) titers in the lung.

A series of additional factors, including exposure to environmental allergens (such as animals, moulds, pollens and mites), cold, exercise, air pollution and drugs can worsen AHR and other aspects of the disease. For a long time, it was believed that airway infection in infancy and early childhood was protective against the development of atopic asthma. For example, a study conducted by Ball *et al*.[3] showed that exposure of young children to other children at day care, or to older children at home, protected against the development of asthma and frequent wheezing later in life. However, over the past decade, this concept has been progressively refined in light of the growing body of evidence that suggests most acute asthma exacerbations are caused by respiratory viral infections as well as the resulting innate immune response.[4-10] In fact, respiratory tract viral infections are associated with 60% of asthma exacerbations in adults and up to 80-85% in children.[11,12] Depending on the different factors (age, gender and race), rhinovirus (RV), coronavirus, influenza virus, parainfluenza virus, adenovirus and respiratory syncytial (RS) virus are known to be the most common viruses that trigger wheezing in infants and exacerbate asthma symptoms in older children. These manifestations occur through different mechanisms, including airway inflammation, mucus hypersecretion and bronchial hyper responsiveness.[13-15]

Among the respiratory viruses, influenza A virus (IAV) is a particularly important cause of viral infection-induced exacerbation of asthma as patients with asthma, especially children, are at higher risk of developing influenza and have more severe problems associated with this disease.[14] However, the exact mechanisms by which influenza virus infection causes asthma exacerbation are not fully understood. The airway epithelial cells (AECs) are the primary sites for influenza virus infections. AEC viral infection leads to activation of signaling cascades to initiate expression of cytokines and chemokines.[16-18] Destruction of AECs and a pro-inflammatory immune response are the primary factors that contribute to the inflammatory cell influx and AHR associated with asthma exacerbation. Asthmatic patients have been shown to have higher levels of IL-13 which promotes a series of events including goblet cell formation, increased mucin secretion, profibrotic repair of airway epithelium, and decreased production of interferon gamma (IFN-γ).[19] Furthermore, direct comparisons of normal and asthmatic AECs in various studies revealed that asthmatic AECs display differential expression of genes associated with inflammation, repair, and remodeling[20-22] that could contribute to viral infection-induced exacerbations of chronic asthma.

It is widely accepted that $T_H1$ cytokines such as IFN-γ antagonize allergic diseases mediated by $T_H2$ cytokines. Respiratory infection by Influenza virus has been shown to lead to the production of high local IFN-γ concentrations by $CD4^+$ and $CD8^+$ T cells expressing low to undetectable amounts of *ex vivo* $T_H2$ cytokine.[23,24] Dahl and colleagues have shown that IAV infection provokes a robust IFN-γ response in the lung that leads to the development of strong, $T_H1$-polarizing dendritic cells (DCs).[10] Using a $T_H2$-dependent mouse model of allergen-induced lung inflammation, they also demonstrated that these DCs strengthen subsequent immunity via enhancing both $T_H1$ and $T_H2$ immune cytokines and immunoglobulin production.[10] In a more recent study, Chang *et al.*[25] have shown that IAV can also promote AHR independently of the adaptive immune system, via production of IL-33 in alveolar macrophages, its receptor ST2 (aka IL1RL1) and cells of the non-T cell, non-B cell innate lymphoid type called "natural helper cells".

Association of asthma exacerbations with influenza infection led to development of early intervention strategies for prevention of asthma. In fact, influenza vaccination is recommended for asthmatic patients, particularly children, in many countries,[26] although there is currently little evidence to support this practice. Despite uncertainty regarding the effectiveness of influenza vaccination in preventing influenza-related asthma exacerbations,[27-29] several other studies conducted on both older and young patients with

asthma diagnosis suggest a protective effect of influenza vaccination on asthma exacerbations.[26,30-33] Influenza infection is a frequent cause of hospitalization of asthmatic patients; thus, regardless of the controversy, patients with asthma will certainly receive the most benefit from influenza vaccination.

**Influenza Virus Replication Process**

Influenza virus attaches to neuraminic acids on the surface of AECs to initiate infection and replication[34] (Figure 1). Human influenza viruses preferentially bind to α2,6 linkage (SAα2,6Gal), whereas avian influenza viruses mostly bind to sialic acid with an α2,3 linkage.[34] Although there remains some debate about how many viruses enter cells, clathrin-mediated endocytosis has been the accepted model for influenza virus entry.[35] However, a non-clathrin, non-caveolae-mediated internalization mechanism has also been described for influenza viruses.[35] Influenza viruses are generally thought to require low pH to fuse with endosomal membranes for effective uncoating. Fusion occurs in three general steps: (1) Influenza hemagglutinin (HA) is first cleaved into HA1 and HA2 subunits; (2) the low pH environment induces a conformational change in the HA subunits to expose the fusion peptide at the N-terminus of HA2; (3) the transmembrane domain of the HA2 (in the viral membrane) and the fusion peptide (inserted into the host endosomal membrane) are in juxtaposition in the low pH-induced HA structure.[34,36]

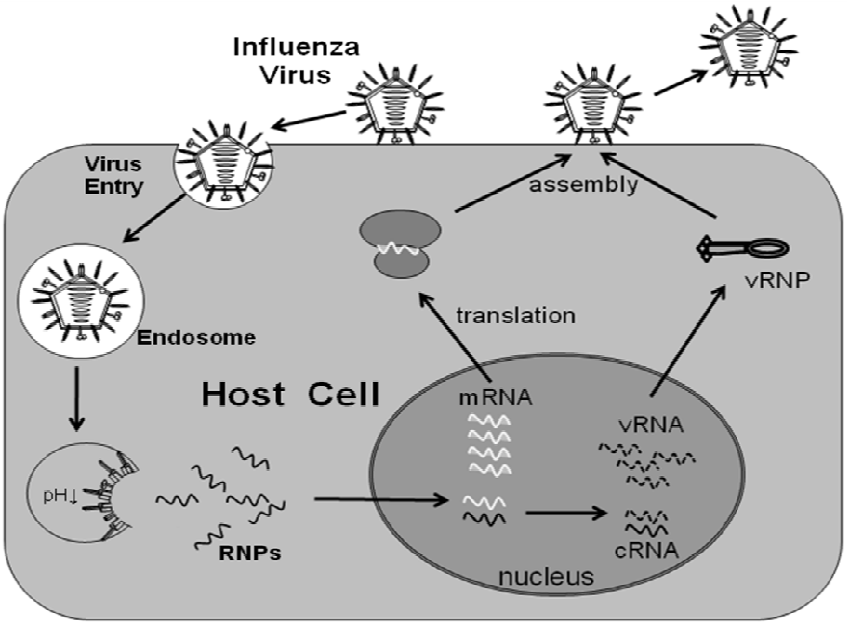


**Figure 1. Influenza virus life cycle. Influenza virus binds to sialic acid molecules on the surface of host cells (epithelial cells lining the respiratory tract) using glycoprotein HA spikes on the virus envelope. This triggers the formation of clatherin coated pits and internalization into endosomes. Inside the endosome, the virus is exposed to a low pH, which triggers a conformational change in the HA protein that leads to fusion of the viral and endosomal membranes. The low pH also triggers the flow of protons into the virus via the M2 ion channel, thus dissociating the vRNPs from M1 proteins, resulted in release of the RNPs into the cytoplasm. The vRNPs are transported into the nucleus where the viral polymerase initiates viral mRNA synthesis. Viral mRNAs are then transported to the cytoplasm for translation into viral proteins. The viral polymerase is also responsible for vRNA synthesis via two steps of replication: (–) vRNA → (+) cRNA → (–) vRNA. The nucleoprotein molecules are then deposited on the cRNA and vRNA during RNA synthesis to make vRNP complexes which are subsequently transported to the cytoplasm. New genes and proteins then come together at the cell membrane, where they are wrapped in a protein layer and encased in a lipid envelope as infectious virus particles and released into the extracellular environment to find new cells to invade.**

One of the characteristics of the influenza virus life cycle that is unusual for an RNA virus is its dependence on nuclear functions. All influenza viral RNA (vRNA) synthesis occurs in the nucleus. In the nucleus, the incoming negative-sense vRNA is transcribed into mRNA by a primer-dependent mechanism. Viral mRNAs are capped and polyadenylated, unlike vRNA. The viral mRNAs are exported from the nucleus and translated to produce viral proteins. Some proteins are shuttled through the ER and Golgi to be glycosylated and inserted into the plasma membrane where viruses will eventually bud during maturation. Many proteins, including the polymerase subunits, are trafficked to the nucleus. Genomic replication then occurs by a two-step process. First, a full-length, positive-sense copy of the vRNA is made that is referred to as complementary RNA (cRNA). The cRNA is then used as a template to produce more vRNA. All of these reactions, vRNA→mRNA, vRNA→cRNA, and cRNA→vRNA are catalyzed by the same viral polymerase complex (PB1, PB2, and PA).

Influenza virus mRNA synthesis requires a 5' capped primer, which it steals from host pre-mRNA transcripts to initiate its own mRNA synthesis.[37] The vRNA serves as a template for both mRNA and cRNA synthesis, and yet the means of initiation and termination for the generation of these two molecules are quite different. In contrast to mRNA synthesis, initiation of cRNA synthesis occurs without a capped primer. The cRNA molecules are full-length complementary copies of vRNA, and newly synthesized vRNAs are encapsidated with NP.[37] It has been proposed that NP encapsidation controls the switch between mRNA and cRNA synthesis.[37] In support of this hypothesis there are observations that viral replication depends on *de novo* protein synthesis, and that free NP has been shown to be required for production of full-length cRNA.[37] A new model has recently been proposed that disputes the existence of a switch, instead suggesting a stabilization role for NP and the polymerase.[37] In contrast to earlier reports, this study claims that the incoming polymerase is able to synthesize both mRNA and cRNA, but newly synthesized cRNAs get degraded. cRNA degradation is only inhibited when there is a sufficient pool of polymerase and NP to encapsidate the cRNA and protect it; therefore, at early times post infection there is a bias toward mRNA accumulation.[37] Thus, exactly how the viral polymerases switch from transcription to replication is still under debate and the molecular details remain to be elucidated. The positive-sense cRNA serves as a template for the synthesis of negative-sense genomic vRNA. As with cRNA synthesis, this reaction also occurs via a primer-independent mechanism and generates full-length products.[37]

NEP/NS2 is responsible for recruiting the export machinery and directing export of the ribonucleoprotein (RNP) complex.[38] Correct assembly and packaging of a full complement of RNA genome segments is a requirement for a fully infectious virion. The precise mechanism of packaging the eight vRNA segments is not well understood. Influenza viruses assemble and bud from the apical plasma membrane of polarized cells[39] (e.g., lung epithelial cells of the infected host). Individual viral envelope proteins are seen to accumulate at the same polar surface where virus budding occurs, suggesting that they determine the maturation site. The enzymatic activity of the NA protein is required to remove the sialic acid and release the virus from its host cell[36] to produce a mature, infectious virus.

**Autophagy Pathway**

Autophagy is an active physiological mechanism to maintain normal cellular function in different species.[40] Autophagy targets long-lived dysfunctional organelles and proteins, then directs them to lysosomes for final digestion.[41] Autophagy is divided into three distinct forms: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy.[40] A variety of stress stimuli including long term starvation, exposure to cytotoxic compounds, or oxidative stress can lead to CMA activation which selectively degrades cytosolic proteins in lysosomes.[42] The exact molecular mechanism that triggers microautophagy remains unknown. However, GTP hydrolysis and calcium ion are considered major initiators of this event in yeast.[43] Macroautophagy (referred to here as autophagy) degrades the bulk of damaged cytoplasmic organelles. Autophagy includes mitophagy (mitochondrial autophagy), ribophagy (ribosomal autophagy), pexophagy (peroxisome autophagy), ER-phagy (endoplasmic reticulum autophagy), aggrephagy (protein aggregate autophagy) and lipophagy (fat autophagy).[40]

Autophagosomes are the major particles that are formed and processed during the autophagy pathway. An autophagosome includes a double-membrane vesicle destined for protein and organelle (cargo) degradation which finally fuses to lysosomes to form autophagolysosomes.[44] Autophagosome formation requires the activity of autophagy-related genes (*ATG* genes) which control Atg protein expression.[45] It was shown that mTOR (mammalian target of rapamycin) inhibits autophagy by restraining the kinase activity of UNC-51-like kinase (ULK).[46] mTOR complex1 (mTORC1) contains mTOR catalytic subunit (raptor/ GβL/PRAS40/ deptor) and phosphorylates ULK1 in the absence of amino acid and growth factor signals [1]. ULK1 Ser/Thr protein kinase, Atg13, and FIP200 (FIP200 is the mammalian homolog of the yeast Atg17) form the ULK1 complex[48-50] and regulate autophagy through phosphorylation of Atg13 and FIP200.[51]

The Beclin 1 complex can also trigger autophagy via c-JUN NH 2–terminal kinase 1 (JNK1), and death-associated kinase (DAPK).[52] Beclin 1 is a platform protein and its complex with class III phosphatidylinositol 3-kinase (PI3K) has a key regulatory role in nucleation and assembly of the initial phagophore membrane.[53,54] Beclin1 interaction with Atg14, UVRAG (ultra-violet radiation resistant gene), and AMBRA (activating molecule in beclin 1-regulated autophagy) promotes autophagy,[55] while Beclin 1 interaction with UVRAG and RUBICON (RUN domain and cysteine rich domain containing) inhibits autophagy.[53,56] Upon autophagy stimulation, LC3βII is conjugated to the polar head of phosphotidylethanolamine (PE) and initiates autophagosme formation.[40] Atg9 is recruited by Atg1-Atg13 signaling complex and plays an important role in expanding the autophagosome precursor.[57] Autophagosomes are labeled by different prominent markers and later are directed to lysosomes and recycled.[52] A summary of the autophagy pathway is illustrated in Figure 2.

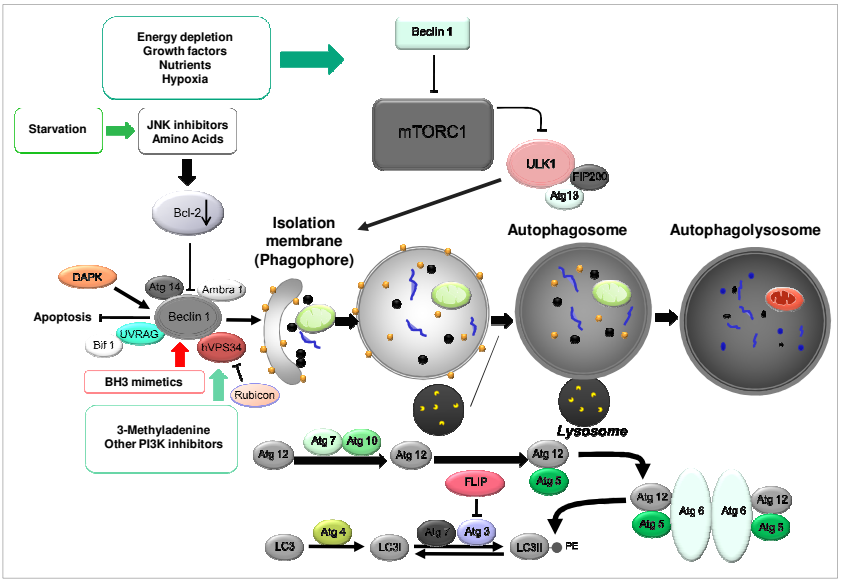


**Figure 2. A schematic summary of the autophagy machinery. Autophagy pathway is tightly regulated because too little or too much activation can be harmful for the cell. The mTORC1 plays a central role in negative regulation of autophagy and is inhibited by the kinase activity of ULK1 complex. Thus, inhibition of mTORC1 strongly induces autophagy. In this pathway, growth factor depletion, essential amino acid deficiency, hypoxia, and low energy levels inactivate mTORC1, cleave ULK1 complex, and trigger autophagy. During starvation, JNK1 is activated which can subsequently phosphorylate Bcl-2, leading to activation of VPS34-Beclin 1-class III PI3-kinase complex which regulates autophagy initiation. Furthermore, the Atg5-Atg12-Atg16 and LC3 conjugation system participates in autophagosome membrane formation and elongation. The Atg5-Atg12-Atg16 complex can conjugate LC3-I to the polar head of the phosphotidylethanolamine (PE) to produce LC3-II (LC3 lipidation). The whole autophagy system is an active flux that directs the autophagosome to lysosomes and finally produces autophagolysosomes to remove unwanted or damaged organelles and cellular debris.**

**Role of Autophagy Pathway in Influenza Virus Infection**

In addition to its cellular homeostasis function, the autophagy pathway is implicated as a central component of antimicrobial host defense against diverse pathogenic infections and not surprisingly, to counteract this mechanism, many pathogens have evolved to evade, subvert, or use autophagy for their own benefit.[58-62] Autophagy has also been shown to be involved in the replication of influenza virus.[59,63-65] However, the exact role of autophagy in IAV pathogeneses remains to be elucidated. Influenza virus is an enveloped virus that uses the low pH of endosomes and lysosomes to uncoat and release the RNPs.[66,67] The lysosomal acidic pH is also required for optimal lysosomal enzyme activity as well as autophagosome and lysosome fusion for completion of the autophagy pathway.[41] According to the Zhou *et al*.,[59] infection by IAV causes an increase in the formation of autophagosomes in mammalian cells. Inhibition of autophagy with pharmacological inhibitors, or by LC3 and Beclin 1 silencing, reduced the titer of virus and autophagosome formation,[59] suggesting a regulatory role for autophagy in influenza virus replication.

Autophagic degradation and recycling in mammalian cells requires autophagosome formation followed by autophagosomes-lysosome fusion. Gannage and colleagues experimentally demonstrated that infection of epithelial cells by IAV inhibits the fusion of autophagosomes with lysosomes, thereby preventing the degradation of autophagosome.[63] In this study, the authors transfected the epithelial cells with different IAV proteins such as polymerase basic 2 protein (PB2), nucleoprotein (NP), non-structural protein 1 (NS1), matrix protein 1 (M1), matrix protein 2 (M2) and hemagglutinin (HA). Only the M2 protein transfection was able to induce autophagosome formation and block its fusion with lysosomes,[63] indicating that M2 protein alone is sufficient to inhibit the process of autophagy. In line with these results, silencing of M2 protein expression during viral infection, or infecting the cells with viruses that lack M2, revert the autophagosome accumulation and allow vesicle degradation.[63] These results suggest that IAV M2 protein is essential for regulating host cell autophagy.

Law and colleagues[65] also showed that infection of human blood macrophages with avian influenza virus H9N2/G1 and H1N1 swine-origin influenza virus (S-OIV) leads to autophagy activation. In this study, H9N2/G1 virus was shown to be the most potent inducer and S-OIV was the weakest inducer of autophagy when compared to other viruses. This observation may suggest that different IAV strains differentially affect the autophagy pathway. Furthermore, the authors showed that autophagy induced by these influenza viruses helped increase the production of C-X-C motif chemokine 10 (CXCL-10) cytokine and interferon alpha (IFN-α),[65] providing new evidence that virus-induced autophagy can regulate cytokine production.

Virus-induced autophagy by avian IAV H5N1 in mouse embryonic fibroblasts (MEF) cells was shown to be due to the suppression of mammalian target of rapamycin (mTOR) pathway,[68] while autophagy in the human epithelial cells was through pathways involving AKT, tumor suppressor protein TSC2 and Mtor.[69] In the latter study, Sun and co-workers experimentally showed that H5N1 HA was primarily responsible for stimulating autophagy.[69] Additionally, they found that treatment of influenza-infected mice with drugs that inhibit autophagy pathway significantly increased the survival rate of mice and ameliorated the acute lung injury and mortality caused by H5N1 infection.[69] Reflecting on the research done so far on influenza viruses, it clearly provides evidence that these viruses induce autophagy upon infection in human cells. However, more evidence is needed in order to understand their mechanism of action in relation to autophagy.

**Apoptosis Cell Death**

Programmed cell death (PCD) is an essential physiological process involved in development, aging and tissue homeostasis which maintains normal cellular fate in different organisms.[70] Apoptosis (PCD I) includes unique biochemical and morphological features such as plasma membrane blebbing, pyknosis, shrinkage and decreased cell volume.[71,72] Apoptosis, in contrast to necrosis, does not induce inflammation since apoptotic cells do not release their cellular contents into the surrounding interstitial tissue and are quickly engulfed by macrophages or adjacent normal cells.[73,74] The regulatory mechanisms of apoptosis are highly controlled at different levels including by death

receptors, caspases, mitochondria and Bcl-2 family proteins.[75] External and internal signals can induce apoptosis (extrinsic and intrinsic pathways) in many cell types.[76]

Caspases, (or Cysteine-Aspartic Protease enzymes), and their activation is considered one of the fundamental components of the mammalian apoptotic pathway.[76] Caspases exist as inactive pro-enzymes (zymogens) that undergo proteolytic processing and are cleaved to an active form during apoptosis.[77] Caspases are classified into two groups based on their order of activation: 1) the initiator caspases (i.e. caspase-2, -8, -9, and -10), and 2) the effector (executioner) caspases (i.e. caspase-3, -6, and -7). Initiator caspases are activated upon extrinsic (cell death receptors) or intrinsic (mitochodrial) stimuli that lead to activation of executioner caspases.[78,79]

Extrinsic or death receptor-dependent apoptosis pathway can be initiated through the ligation of death receptors (Fas, DR4, DR5, TNF-R1) by their specific ligands (e.g., FasL, APO-2L, TRAIL, and TNF).[76] Once a death ligand binds to its corresponding receptor, endogenous adaptor proteins such as FADD or TRADD are recruited and apoptotic signals can conscript adaptor molecules to their death domains which will trigger the activation of apical initiator caspases (e.g., caspase-8). Active caspase-8 affects mitochondria via truncated BID and causes mitochondrial initiator caspase (caspase-9) activation.[80,81] All of these events lead to effector caspase activation (caspase-3, -7, -6) [2], that can cleave different substrates such as cytokeratins, PARP, plasma membrane cytoskeletal protein (alpha fodrin) and subsequently provoke morphological and biochemical aspects of apoptosis.

The mitochondria-dependent pathway is triggered following exposure to stresses such as cytotoxic drugs, ultraviolet (UV) radiation, and free radicals which lead to DNA damage.[72,82-84] Later stress can activate pro-apoptotic Bcl-2 family (Bax/Bak) and ultimately lead to caspase-dependent or -independent apoptosis.[76,85] Anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-XL) counteract pro-apoptotic proteins and can delay or inhibit apoptosis.[86] A summary of apoptosis pathways is shown in Figure 3.

**Role of Apoptosis Pathway in Influenza Virus Infection**

Apoptosis is a key player in many viral infectious diseases, including influenza virus infection.[87] However, the exact role of apoptosis in viral infections is not fully understood yet. Two controversial and opposed hypotheses concerning the role of apoptosis in viral infections have been proposed; one considers apoptosis as a host cell defense mechanism against viral infection[88] whereas the other one proposes that viruses hijack host cell apoptosis machinery and use it for its own replication process.[89-93] Several *in-vivo* and *in-vitro* studies highlight the importance of influenza virus-induced apoptosis mechanisms in different models.[93-95] Different influenza virus strains contain a variety of proteins including M1, NS1, and PB1-F2 proteins, which are shown to be involved in apoptosis induction or inhibition.[96]

Microtubules are an important component of the cellular cytoskeleton structure within the cell's cytoplasm.[97] The Influenza virus NS1 protein was shown to interact with tubulin and affects its polymerization.[98] This could arrest the infected cells in G2/M phase and further affect Bcl-2 phosphorylation which triggers apoptosis cascade in infected cells.[98] Moreover, it has been recently shown that agents that interfere with tubulin polymerization can also induce apoptosis.[99] Several studies investigated the role of pro- and anti-apoptotic Bcl-2 family proteins including Bcl-2, Bax, Bak and BAD in influenza virus infection. Over-expression of anti-apoptotic protein, Bcl-2 decreased influenza virus virulence[100] while Bax and BAD activation are necessary for successive viral replication.[93,100] In a recent study conducted by our group, we found that influenza virus infection induces phosphorylation of BAD at residues S112 and S136 in a temporal manner and that virus-induced cytopathology and cell death are considerably inhibited in BAD knockdown cells.[93] In contrast, the pro-apoptotic protein Bak has anti-viral effects and is significantly down-regulated during IAV infection.[100] Bax expression was also shown to be negatively regulated via PI3K/Akt/JNK pathway in virus infected cells,[101] which further affects influenza virus infection in the host cells. These studies indicate the importance of Bcl-2 family members in influenza virus infection and demonstrate that further study is necessary to address the exact mechanisms involved in these processes.

Initiator and effector caspases are involved in apoptosis and their activation is one of the essential hallmarks of apoptosis.[76,102] It has been shown

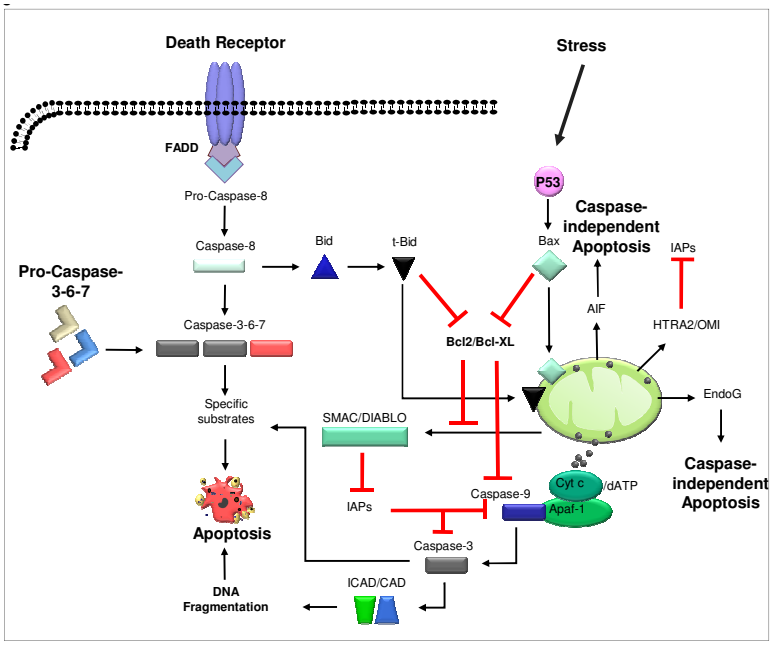


**Figure 3. Schematic representation of extrinsic and intrinsic apoptotic pathways. Cell death ligands (e.g., FasL, APO-2L, TRAIL, TNF) bind to their respective death-receptors (e.g., Fas, DR4, DR5, TNF-R1) and initiate pro-caspase-8 activation by recruiting FADD. Once caspase-8 is activated, it can directly initiate the cleavage of effector caspases, such as caspase-3, -6, and -7, which are involved in the core apoptosis pathway. Moreover, caspase-8 truncates Bid, which later induces the intrinsic (mitochondrial) pathway. The mitochondria pathway can be directly initiated by a variety of stress signals. Stress signals initiates DNA damage and p53 phosphorylation, which subsequently triggers many apoptotic events and finally causes mitochondrial damage and cytochrome c release. Cytosolic cytochrome c participates in apoptosome formation (in the presence of dATP, and Apaf1) and activates caspase-9 which later activates caspase-3. Caspase-3 selectively cleaves CAD that causes nuclear fragmentation and also chromatin condensation. Damaged mitochondria may also release other pro-apoptotic proteins such as Smac/Diablo, Omi/HtrA2 (caspase dependent), AIF, and Endo G (caspase independent) that separately can participate in apoptosis propagations.**

that influenza virus NS1 protein activates NF-κB and induces INF-α and INF-β, which afterward activate the caspase cascade and induce apoptosis in infected cells.[103,104] Using the human alveolar epithelial cell line A549, Yan *et al.*[105] demonstrated that the 2009 pandemic H1N1 A/Beijing/501/2009 can induce caspase-3-dependent apoptosis.[106] Avian H5N1 influenza virus has also been shown to promote TNF-related apoptosis-inducing ligand (TRAIL) in human monocyte-derived macrophages (MDMs). In the later events, TRAIL caused caspase-10 activation with subsequent BID truncation and apoptosis inducing factor (AIF) release form mitochondria which ultimately leads to apoptosis in these cells. Z-VAD-fmk (pan caspase inhibitor) inhibits all of these events in H5N1 infected MDM cells.[105] Yang *et al*. showed that caspase-dependent apoptosis is involved in influenza A/chicken/Hubei/489/2004 virus infected MDCK cells and that virus infection is also regulated by apoptosis in these cells.[107] As was mentioned in previous sections, activated caspases have several targets. It has been shown that activated caspase-3 can cleave histone deacetylase 6 (HDAC6) in IAV-infected MDCK cells and cause further damage in these cells.[108]

Regulation of apoptosis in many ways has been shown to effect virus replication in the host cells. For instance, microRNAs (miRs) have been shown to control apoptotic signaling through numerous signaling pathways including Bcl-2 family proteins,[109-110] small Rho GTPase[111] and *ATF1* gene expression.[112] Virus infection also has been shown to alter the expression of cellular miRs,[113,114] which could affect virus replication

in the host cells. In a recent study, it was shown that influenza virus infection affects miR-29c and down-regulates Bcl2-L2 expression, which leads to apoptosis promotion in A549 cells.[115] Antioxidant compounds are also known to increase the strength of the cellular defense against multiple apoptotic stimuli and augment cell survival mechanisms.[116-118] A study conducted by Mata and colleagues showed that infection of host cells with IAV and simultaneous anti-oxidant agent treatment decreased influenza virus replication and apoptosis induction.[119]

Natural Killer (NK) cells are a major part of the host innate immune system against pathogens including viruses.[120] It has been reported that influenza virus could directly induce apoptosis in NK cells and probably facilitate viral transmission which could smooth the progress of viral pathogenesis.[120] Influenza virus could also induce apoptosis in neutrophils of the host via an increase in Fas protein expression in these cells.[121] In a more recent study, virus-induced apoptosis in neutrophils has been shown to induce innate immunity dysregulation in the host cell and increase the pathogenesis of influenza virus.[122] As evident from the literature review of the researches done so far on influenza viruses and apoptosis, there appears to be a complex in virus-infected host cell responses in relation to apoptosis pathway. This is very logic that influenza viruses evolve to use an economic way to control efficient replication, thus virus-induced apoptosis at some point during the virus life cycle may act as a proviral signaling pathway. Additional studies are warranted to shed further light on the role of apoptosis in influenza virus infection.

**Endoplasmic Reticulum Stress and Unfolded Protein Response**

The endoplasmic reticulum (ER) serves as a protein-folding machinery and plays critical functions in maintaining normal cellular activities.[123] Imbalance between the ER protein folding capacity and cellular protein demand, resulting in accumulation of mis-folded protein in the ER, leads to the unfolded protein response (UPR).[124,125] Several stimuli can induce ER stress, such as disruption of $Ca^{2+}$ homeostasis, various metabolic abnormalities, toxins, inhibition of proteins glycosylation, spontaneous errors during transcription and translation, genetic mutations, virus or bacterial infection, and hypoxia.[126,127] ER stress transmembrane receptors, pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 alpha (IRE1α) detect the aggregation of unfolded proteins and then induce UPR to maintain normal ER function.[123,128] UPR arms are docked and kept inactive by immunoglobulin heavy chain binding protein (BiP).[123] However, in stressed conditions, BiP can bind to lumenal misfolded proteins, which leads to PERK, IRE1α and ATF6 activation.[124]

Upon activation, PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2α), thereby shutting off protein translation.[123] However, under ER stress-related conditions, ATF4 expression is up-regulated and affects cellular redox status and regulates apoptosis mechanisms.[124] In addition to eIF2α, PERK also phosphorylates Nrf2, an antioxidant response transcription factor, and increases its stability.[129,130] IRE1α induces X-box binding protein-1 (XBP-1) mRNA splicing,[131,132] which is also essential for efficient protein folding, maturation, and degradation in the ER.[133,134] Prolonged activation of IRE1α triggers Jun N terminal kinases (JNKs) signaling pathway which is linked to many forms of stress-regulating gene expression.[135] IRE1α can also recruit the adaptor molecule TNF-receptor-associated factor 2 (TRAF2) as well as the apoptosis signal-regulating kinase (ASK1), which leads to caspase activation and cell death.[136] Caspases-12 also is implicated in ER stress-induced death signals and promotes activation of effector caspases.[137]

ATF6 is the third arm of the UPR which is processed by serine protease S1P and the metalloprotease S2P after translocation to the Golgi apparatus.[123] Cleaved ATF6 subsequently moves to the nucleus, where it can induce expression of genes with an ER stress response element (ERSE) in their promoter region.[138] ATF6-targeted genes include ER chaperone proteins CHOP, XBP-1, ERp72, and PDI.[139] A summary of ER-stress and UPR pathways is illustrated in Figure 4.

**UPR and Influenza Virus Infection**

In the past decade, many researchers have addressed different aspects of UPR in various pathological conditions including cancer, inflammation and metabolic disorders.[140-143] UPR and ER-stress are also involved in many virus infections including hepatitis B virus, hepatitis C virus, Japanese encephalitis virus, Enterovirus 71, and Moloney murine leukemia virus (MoMuLV)-ts1.[144-146]

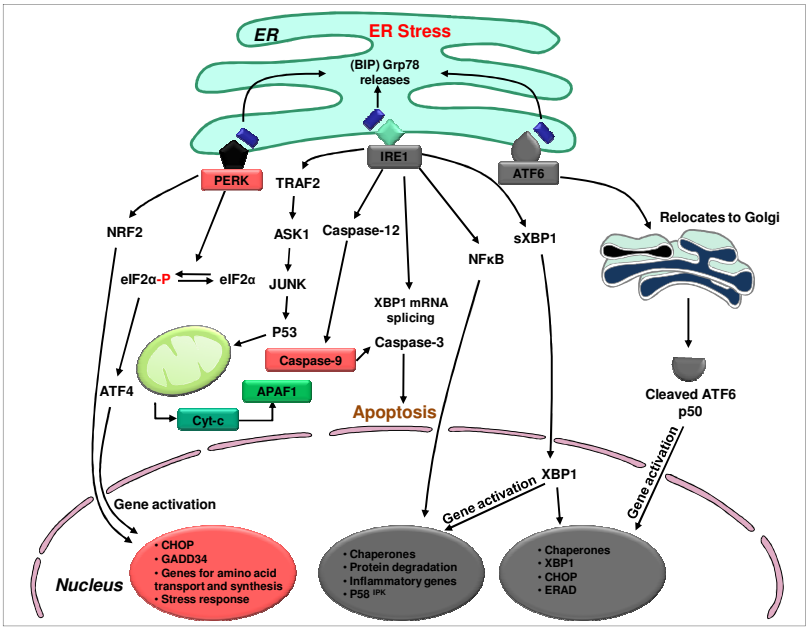


**Figure 4. The molecular events of ER stress. In stressed cells, the ER chaperone BiP (GRP78) releases from UPR proteins (e.g. PERK, ATF6, and IRE1) and facilitates their activation. Upon activation of PERK, eIF2α is phosphorylated and block protein synthesis. However, selected mRNAs like ATF4 are translated in the presence of phosphorylated eIF2α and activate genes encoding ATF3, CHOP, and GADD34 expression, which also participate in translational inhibition. IRE1 activation results in the splicing of XBP-1 mRNA in the cytoplasm, leading to its nuclear translocation and transcription of UPR target genes. TRAF2 and ASK1 are also recruited by IRE1 and activate JNK and NFkB. The third UPR sensor (ATF6) translocates to the Golgi apparatus where it is cleaved by S1P and S2P proteases. After being cleaved, ATF6 moves to the nucleus and targets ER chaperone genes (CHOP, XBP-1, ERp72, and PDI).**

In many cases, UPR is involved in viral pathogenesis events in the host cells. For instance, Japanese encephalitis virus, bovine diarrhea virus, tula virus, severe acute respiratory syndrome coronavirus (SARS-CoV), and West Nile virus have been demonstrated to induce their apoptotic effect via UPR induction in their host cells.[147-150]

It has been recently reported that IAV infection activates certain arms of UPR in the lung epithelial cells.[151] Hassan *et al.* reported that IAV infection induced IRE1 activation with subsequent XBP-1 splicing while it did not affect PERK activation in the lung epithelial cells.[151] This observation was further confirmed using specific IRE1 inhibitors which inhibited IAV replication in these cells.[151] In another study conducted by Ruberson *et al.*,[152] the authors reported that IAV infection induces ER-stress in murine primary tracheal epithelial cells (MTECS) via ATF6 and endoplasmic reticulum protein 57-kD (ERp57) activation, but not C/EBP homologous protein (CHOP). They also reported that IAV mediated-apoptosis in these cells is caspase-12 dependent, which is another hallmark of ER-stress in the infected cells.[152]

Human myxovirus resistance gene A (MxA), which is responsible for the antiviral activity against a variety of RNA viruses, including influenza virus, has been shown to be responsible for ER stress-induced events, such as *BiP* mRNA expression and *XBP-1* mRNA processing in IAV infected cells.[153] Furthermore, IAV mRNA translation may also need the enrollment of $P58^{IPK}$, the cellular inhibitor of PKR, an interferon-induced kinase that regulates the eukaryotic translation initiation factor eIF2α [3]. It has been previously shown that $P58^{IPK}$ regulates influenza virus mRNA translation and infection through a PKR-mediated mechanism, which is independent of PERK.[154]

**Closing Remarks**

Reducing asthma rates, and controlling its symptoms in patients, are one of the most important aims for many respiratory-field scientists. One of the factors that might be involved in both etiology and recurrence of asthma is respiratory tract infectious diseases, including influenza. The present review paper addresses the involvement of apoptosis, autophagy, and UPR in influenza virus replication and cell cycle. Most of the findings are controversial but careful consideration highlights an important point; that these pathways could positively or negatively regulate viral replication. However, the time points at which measurements are taken, or when interventions are introduced, are the most critical aspects that should to be considered. Apoptosis, autophagy, and UPR are major host cell responses to viral infection. Therefore, it is crucial that these events be studied in both early and late time points post infection. Several chemical modulators of these pathways have been invented and are currently in use for different diseases. Thus, these pathways and their modulators could potentially be a future target in modern and novel influenza virus infection therapeutic strategies for controlling the rate and symptoms of asthma disease.

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