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Innate immune evasion strategies of influenza viruses

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Abstract

Influenza viruses are globally important human respiratory pathogens. These viruses cause seasonal epidemics and occasional worldwide pandemics, both of which can vary significantly in disease severity. The virulence of a particular influenza virus strain is partly determined by its success in circumventing the host immune response. This article briefly reviews the innate mechanisms that host cells have evolved to resist virus infection, and outlines the plethora of strategies that influenza viruses have developed in order to counteract such powerful defences. The molecular details of this virus–host interplay are summarized, and the ways in which research in this area is being applied to the rational design of protective vaccines and novel antivirals are discussed.

Keywords

antivirals; cell signaling; influenza virus; interferon; NS1; pandemic; vaccines

Influenza viruses

Influenza viruses are classified within the *Orthomyxoviridae*, a family of enveloped viruses that all contain a segmented, single-stranded, negative-sense RNA genome [1]. Members of this family that regularly infect humans include influenza virus types A, B and C [1,2]. Influenza A viruses (Box 1) also naturally infect a variety of other animal species, including birds, pigs, horses, seals, whales, mink, giant anteaters, cats and dogs [3–5], whilst influenza B and C viruses appear predominantly (but not exclusively) limited to humans (reviewed in [4]). Disease caused by influenza viruses is often an acute, highly contagious, respiratory illness

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that usually affects the upper respiratory tract (i.e., the nose, throat and bronchi). Infection is characterized by the sudden onset of fever, muscle pain, headache and severe malaise, together with a nonproductive cough, sore throat and nasal inflammation (reviewed in [4]). Sometimes influenza virus infection also affects the lower respiratory tract, causing primary viral pneumonia. In addition, secondary bacterial pneumonia has been associated with influenza virus infection. However, such severe cases are usually limited to either the very young or elderly, or those with chronic predisposing health conditions.

Seasonal influenza

In temperate climates, 'seasonal influenza' occurs mostly during the winter months, a phenomenon likely due to the physical susceptibility of viruses to temperature and humidity [6]. Such annual epidemics are caused by newly circulating influenza A and B virus strains generated by the process of 'antigenic drift' (Box 2), and seriously affect an estimated 3–5 million people worldwide (reviewed in [7,301]). In general, mild cases of infection are naturally cleared by the combined effects of adaptive immune responses together with powerful innate immune responses. Although most people recover from influenza within 1–2 weeks without requiring medical treatment, infections in certain at-risk groups can result in pneumonia, hospitalization and death (reviewed in [4]). Thus, despite the huge benefit of targeted vaccination programs in many countries, seasonal influenza outbreaks still account for 250,000–500,000 deaths every year worldwide [301].

Pandemic influenza

The remarkable zoonotic capacity of influenza A viruses (but not influenza B viruses), combined with the ability of segmented viruses to reassort their genomes, sometimes leads to the generation of antigenically novel viral strains (see antigenic shift; Box 2) that can replicate efficiently in humans, but to which very little immunity preexists in the population. In addition, it is possible for novel influenza A virus subtypes to emerge directly from animal reservoirs without reassortment. Occasionally, such a virus is able to transmit between people and affects 20–40% of the world's population in a single year [7,8]. Such unpredictable, yet periodic, influenza pandemics occurred three times in the 20th century: in 1918–1919, 1957–1958 and 1968 (reviewed in [4,9]). The 1918 (Spanish flu) pandemic was the most severe, and although the precise origins of this virus are unclear, it may have been directly transmitted to humans from an avian source [9]. The 1918 influenza A virus is estimated to have clinically affected approximately 500 million people (approximately a third of the world's population at the time), and contributed to more than 40 million deaths (reviewed in [8,9]). The pandemics of 1957 (Asian flu) and 1968 (Hong Kong flu), which were caused by reassortment events, had much lower, yet still significant, mortality rates [4,7]. A characteristic of all these pandemics (although most dramatically with the Spanish flu) is their association with severe disease progression even in young, healthy adults (reviewed in [8]). Since 1997, repeated lethal human infections (predominantly in southeast Asia) caused by highly pathogenic H5N1 avian influenza A viruses have led many to believe that a similar virus will eventually cause a devastating human pandemic (reviewed in [10]). Meanwhile, the apparent direct emergence from pigs, and rapid worldwide (pandemic) dissemination in humans, of an antigenically novel H1N1 influenza A virus (Swine-Origin 2009) is of significant current concern [11,12].

Box 1. Influenza A viruses

- Influenza A virus particles (virions) have a typical diameter of 80–160 nm, and are pleomorphic in shape.
- Virions consist of a lipid envelope (derived from the host plasma membrane) out of which protrude two surface glycoproteins: hemagglutinin and neuraminidase.

- There are 16 different hemagglutinins and nine different neuraminidases, and reassortment of these two antigens determines the influenza A virus subtype (e.g., H1N1 or H3N2).
- Embedded within the lipid envelope bilayer is the viral integral membrane ion channel protein, M2, whilst the viral matrix protein (M1) lies underneath the envelope and structurally supports the particle.
- Contained within this matrix shell is the viral RNA genome, which (for influenza A viruses) consists of eight single-stranded, negative-sense RNA segments (negative-sense refers to the genome being complementary to mRNA, which by convention is termed positive-sense)
- Each strand is wrapped up in multiple copies of the nucleocapsid protein, and is associated with three proteins responsible for transcription and replication: polymerase subunit basic (PB)1, PB2 and polymerase subunit acidic (the viral polymerase complex).
- Each nucleocapsid protein-encapsidated RNA segment, together with its heterotrimeric polymerase, constitutes a viral ribonucleoprotein complex. Although consisting of only eight genomic segments, multiple gene-encoding mechanisms mean 11 viral proteins are produced during infection (reviewed in [1]).
- Other than the proteins detailed above, the nuclear export protein, nonstructural protein 1 and PB1-frame 2 are produced. Only nonstructural protein 1 and PB1-frame 2 are not incorporated into the virus particle, and are found only in infected cells. Functions of each viral protein are listed in Table 1.

Virus–host interface

The interplay between viruses and their hosts is of pivotal importance for determining the clinical outcome of infection. Cells respond to virus infection by inducing an innate immune interferon (IFN) response, which is an extremely potent antiviral mechanism for limiting early virus spread (reviewed in [13]). In addition, cytokines and IFN produced during virus infection shape adaptive immune responses, including antibody responses and T-cell activation ([14–20], and reviewed in [21,22]). Nevertheless, in some instances, such powerful responses prove detrimental to the host. For example, infections with highly pathogenic influenza viruses can sometimes result in the excessive and dangerous production of proinflammatory cytokines and IFN. This uncontrolled cytokine production is known as a ‘cytokine storm’, and may contribute to morbidity and mortality during the associated infection [23–30].

In order to replicate efficiently, influenza viruses have evolved multiple strategies to circumvent the ‘first line of defence’ embodied by the IFN system. Below, current knowledge on these critical virus–host molecular interactions are reviewed, and the ways in which work in this area is contributing to the development of novel prophylactic and therapeutic strategies are discussed.

Box 2. Antigenic drift versus antigenic shift

- Antigenic drift is the process whereby mutations accumulate in viral surface glycoproteins (predominantly hemagglutinin), resulting in viruses with slightly different antigenic profiles.

- Antigenic shift occurs when the surface hemagglutinin and neuraminidase segments reassort between different viruses, resulting in a virus with completely new surface glycoproteins.

Host innate immune response to infection

Interferon from chorionic membranes of embryonated chicken eggs was first noted as a naturally produced antiviral substance during the middle of the 20th century by Isaacs and Lindenmann [31,32]. Since its initial discovery, three IFN types have been identified [33], including type I IFN (mainly α/β), type II IFN (γ) and type III IFN (λ). Type II IFN contributes to the establishment of adaptive immune responses [34,35]. Although type III IFN has been shown by several studies to control influenza virus infection [36], the type I IFN response appears most critical for limiting influenza virus replication and thereby ensuring host survival [37–41].

Type I IFN induction

Virus replication results in the synthesis of several types of pathogen-associated molecular patterns (PAMPs). The major influenza virus PAMP is thought to be cytoplasmic viral RNA species that contain triphosphate groups at their 5' ends (as opposed to the 7-methyl guanosine cap structures present at the 5' ends of cellular transcripts) [42]. dsRNA is a widely recognized PAMP produced during infection with some RNA viruses. Although influenza viruses do not appear to produce detectable amounts of dsRNA during infection [42,43], low levels of viral dsRNA might also represent an influenza virus PAMP. Host cells detect the presence of an infecting virus by pattern recognition receptors (PRRs) that recognize the PAMPs and initiate antiviral signaling cascades that ultimately effect an antiviral response (reviewed in [13]). PRRs are divided into several families, including nucleotide-binding oligomerization domain (NOD)-like receptors, Toll-like receptors (TLRs), and retinoic acid-inducible gene-I (RIG-I)-like helicases (reviewed in [44]). Since the initial observation that virus infection induces caspase-1 activation in a cryopyrin/Nalp3-dependent manner [45], the potential contribution of NOD-like receptors to the establishment of innate immune responses against influenza virus infection has been more closely examined [46–48], but this will not be discussed further here.

Toll-like receptors are the most extensively studied family of PRR. They are transmembrane proteins expressed by multiple cell types and are located on either the cell surface (TLR1, 2, 4 and 5), or on cytoplasmic structures such as endosomes (TLR3, 7, 8 and 9; reviewed in [49]). Although most surface TLRs recognize bacterially derived PAMPs, TLR4 has been shown to recognize the fusion glycoprotein of respiratory syncytial virus [50]. It is unknown whether TLR4 also recognizes either of the two influenza virus surface glycoproteins (hemagglutinin and neuraminidase [NA]), but TLR4 signaling during infection with highly pathogenic H5N1 influenza A virus has been reported to contribute towards lung pathology [51]. TLRs are generally necessary for antigen-presenting cells (i.e., macrophages and plasmacytoid dendritic cells) to respond to virus infection by inducing innate immune responses [49]. In particular, TLR7 is critical for influenza virus-induced IFN production by plasmacytoid dendritic cells [52,53].

Retinoic acid-inducible gene-I-like helicases are present in the cytoplasm of many cell types, including conventional dendritic cells, macrophages and pulmonary epithelial cells (the principle target for influenza virus replication) [54]. The RIG-I-like helicase family is primarily comprised of the DExDH box RNA helicases RIG-I, melanoma differentiation-associated gene-5 (MDA-5), and laboratory of genetics and physiology-2 (LGP-2). These PRRs are thought to 'sense' distinct viral cytoplasmic RNA PAMPs produced during infection, and may act either antagonistically or in synergism with one another (reviewed in [13]). Nevertheless,

studies with cells and mice deficient in either RIG-I or MDA-5 suggest that only RIG-I is essential for induction of IFN in response to influenza virus infection [55–58].

Since the first observation of the critical contribution of RIG-I in inducing innate immunity [59], a detailed picture of the signaling pathway that leads from RIG-I activation to induction of IFN has emerged (summarized in Figure 1). RIG-I is comprised of several functional domains, including two tandem amino-terminal caspase activation and recruitment domains (CARDs), a central ATP-dependent helicase domain and a carboxyl-terminal regulatory domain (RD) [56]. In its inactive state, RIG-I exists as a monomer in which the RD is thought to mask the CARDs. Binding of the RD to PAMPs (either dsRNA or cytoplasmic 5'-triphosphate containing viral RNA) results in a conformational change leading to RIG-I dimerization and exposure of the CARDs (reviewed in [60]). Gack *et al.* recently demonstrated that ubiquitination of lysine 172 within the second CARD of RIG-I is essential for IFN production in response to virus infection [61]. This posttranslational modification of RIG-I is performed by the IFN-inducible E3 ubiquitin ligase, tripartite motif (TRIM)25 [61]. Following ubiquitination, RIG-I initiates a signaling cascade that begins with its relocalization to mitochondria, where the exposed, ubiquitinated CARDs of RIG-I associate with the CARD of mitochondrial antiviral signaling adaptor (MAVS; also known as IPS-1/VISA/Cardif) [62]. MAVS functions as an essential scaffolding factor [63] that recruits two multiprotein 'signalosome' complexes consisting of a variety of E3 ubiquitin ligases, additional scaffolding proteins and numerous protein kinases (for recent reviews see [64,65]). The first complex contains TNF-receptor-associated factor 3 (TRAF3), TRAF family member-associated NF- κ B activator (TANK), TBK1, and IKK ϵ , which phosphorylates the transcription factor IFN regulatory factor 3 (IRF-3). The second kinase complex consists of TRAF6, receptor-interacting protein (RIP)1, NF- κ B essential modulator (NEMO), TAK1, IKK α and IKK β , which phosphorylates inhibitor of κ B (I κ B), ultimately leading to NF- κ B activation. Phosphorylated IRF-3, activated NF- κ B and ATF-2/c-Jun all translocate to the nucleus, where they form an enhanceosome complex on the IFN- β promoter and transcribe IFN- β mRNA (Figure 1) (reviewed in [13]).

Type I IFN signaling

After translation, newly synthesized bioactive IFN- β is secreted from the infected cell and engages with either the IFN- α/β receptor (IFN- α/β R) of the same cell (autocrine signaling) or the neighboring cell (paracrine signaling). The IFN- α/β R is a dimeric structure composed of the subunits IFN- α/β R1 and IFN- α/β R2; the cytoplasmic tails of these subunits serve as docking platforms that initiate a signaling cascade in response to IFN- β (Figure 2). This cascade is dependent on coordinated protein phosphorylation and protein–protein interactions involving Tyk2, Jak1, STAT1 and STAT2 [66]. The consequence of this signaling cascade is the generation of a nuclear IFN-stimulated gene factor-3 transcription factor complex (ISGF3), which comprises a heterodimer of phosphorylated STAT1/STAT2 complexed with IRF-9 (reviewed in [13]). Activated ISGF3 stimulates the transcription of over 300 genes that lie downstream of IFN-stimulated response elements ([67] and reviewed in [66]). These gene products establish a general 'antiviral state' within cells that limits virus replication (Figure 2). For influenza viruses, the best-characterised antiviral proteins include: dsRNA-activated protein kinase, PKR (translational repression, [68]); 2'–5' oligoadenylate synthetase (OAS; activator of RNaseL, mRNA degradation [69]); myxovirus resistance gene A (MxA; dynamin-like large GTPase recognising and inhibiting the viral ribonucleoprotein [RNP] structure [70]); viperin (inhibits viral release [71]); and IFN-stimulated gene (ISG)15 (a ubiquitin-like modifier that apparently regulates a number of IFN-stimulated proteins [72,73]).

Influenza virus antagonism of innate immunity

The seminal study by Isaacs and Lindenmann [31] revealed that treatment of chorionic membranes with heat-inactivated influenza virus stimulates the release of an inhibitory substance (IFN) that limits the replication of infectious influenza viruses. In hindsight, these studies also indicated that, unlike heat-inactivated virus, infectious influenza viruses do not efficiently stimulate IFN production. More recent *in vitro* studies using immortalized human lung cell lines have confirmed that, in general, wild-type influenza A viruses are poor inducers of type I IFN [74]. Several further studies have uncovered the many strategies employed by influenza viruses to limit both directly and indirectly the global cellular antiviral state. As outlined below, this is thought to primarily involve viral targeting of the IFN-induction and signaling cascades at multiple levels. Influenza viruses are by no means unique in their ability to limit the IFN response, and in order to replicate efficiently all viruses must be able to counteract these host defences to some extent. How other viruses subvert innate immunity has been reviewed elsewhere [13,55,64].

Nonstructural protein 1 limits IFN production

The influenza A virus nonstructural (NS)1 protein is a multifunctional virulence factor, the major function of which appears to be antagonism of host innate immunity (for an extensive recent review of the structure and functions of NS1 see [75]). This was first demonstrated after reverse genetics techniques allowed the generation of recombinant influenza viruses that either lacked the *NS1* gene [38] or expressed NS1 truncation mutants [76]. Infection of cell cultures and animal models with these viruses revealed that the mutant viruses induced robust IFN secretion from infected cells [77–82]. Furthermore, the importance of NS1-mediated IFN inhibition was confirmed when these mutant viruses were shown to cause significantly reduced morbidity in mice, chickens, swine, horses and macaques [38,78–80,83–86]. Using a mouse model of infection, it has recently been proposed that NS1 expression allows for a brief period of ‘stealth’ virus replication preceeding the onset of host innate and adaptive immune responses [87]. It should be noted that the IFN-antagonistic property of NS1 appears functionally conserved between both influenza type A and B viruses [38,76,88,89].

NS1 inhibits the RIG-I signaling cascade

Initial *in vitro* and cell culture assays revealed that the NS1 protein prevents virus-induced activation and translocation of IRF-3 [90], NF- κ B [91] and ATF-2/c-Jun [92]. Thus, by blocking activation of these individual components of the enhanceosome complex, the NS1 proteins of both influenza A and B viruses can limit RIG-I-mediated transcriptional activation of the IFN- β promoter [42,93–95]. Furthermore, microarray studies have revealed several classes of genes whose transcriptional activation is dependent on activated RIG-I [56] or phosphorylated IRF-3 [96]. Thus, in addition to inhibiting *IFN*- β gene expression, the NS1 protein likely prevents expression of numerous other cellular genes that are also transcriptionally dependent on the RIG-I-mediated signaling cascade.

Biochemical studies initially suggested that IFN inhibition by the influenza A virus NS1 protein requires formation of a complex containing NS1, RIG-I and possibly a viral PAMP (e.g., dsRNA) [42,95]. Such a complex appears to allow NS1 to block activation of the IFN- β promoter even in the presence of a constitutively active version of RIG-I composed only of its CARDs [42,93–95]. However, it is not clear whether NS1 can bind directly to RIG-I [95], and recent data have uncovered further mechanistic details about the nature of this multiprotein inhibitory complex: the NS1 proteins of human, avian and swine influenza A viruses interact with and inhibit the activity of the E3 ubiquitin ligase, TRIM25 (Figure 1) [97], which (as described above) is required to posttranslationally modify RIG-I and thereby stimulate its signaling cascade [61].

Tripartite motif 25 retains several evolutionarily conserved domains that are common among the ten subfamilies in the TRIM family [98], including an amino-terminal ‘really interesting new gene’ (RING) domain that mediates its enzymatic function, central B-boxes and a coiled-coil domain that mediates oligomerization. However, TRIM25 possesses a carboxylterminal splA and ryanodine receptor (SPRY) domain that is not shared by all subfamilies of TRIM proteins [98]. The SPRY domain of TRIM25 was shown to bind the CARDs of RIG-I [61]. TRIM25-mediated ubiquitination of RIG-I in response to virus infection is a complex process involving coiled-coil domain-mediated oligomerization, binding of the SPRY domain to the first CARD domain of RIG-I [61,99], and ubiquitination of lysine 172 within the second CARD of RIG-I by the RING domain of TRIM25 [61]. NS1 was shown to prevent TRIM25 oligomerization by interacting with its coiled-coil domain, thereby preventing TRIM25 ubiquitinating RIG-I [97]. The specificity by which the NS1 protein binds the coiled-coil domain of TRIM25 has not been completely examined and, therefore, whether NS1 interacts with additional members of the TRIM family has yet to be determined. In addition, given that proteins other than RIG-I appear to be substrates for both TRIM25-mediated ubiquitination [100] and ISGylation [101], TRIM25 inhibition by NS1 may have a number of additional consequences during infection. Although it is known that the influenza B virus NS1 protein can also block IFN production pretranscriptionally [89], it is unclear if this NS1 also binds TRIM25. As detailed below, not all functions of the influenza A and B virus NS1 proteins are conserved.

Influenza viruses regulate host cell gene expression

In addition to directly blocking the cytoplasmic signaling cascade that results in *IFN-β* gene transcription, the influenza A virus NS1 protein has also been reported to deregulate general host cell gene expression [102]. This is largely accomplished by NS1 binding two zinc-finger domains of the nuclear 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) [102–105]. This interaction prevents CPSF30 from carrying out its normal cellular function of correctly processing the 3′ ends of pre-mRNAs into mature, polyadenylated mRNAs (Figure 1) [102,106–108]. Biochemical and crystallographic studies of the NS1–CPSF30 complex revealed the regions of NS1 necessary for its interaction with CPSF30 [103,109]. The main interaction site is centered around tryptophan-187, whilst residues including phenylalanine-103 and methionine-106 contribute to stability of the NS1–CPSF30 complex [103,109]. These insights helped explain previous conflicting results, whereby the isolated NS1 proteins from certain naturally occurring and laboratory-adapted virus strains appeared to lack the ability to bind/inhibit CPSF30 [74,103,110]. An additional mechanism that NS1 may utilize to limit gene expression is to block cellular mRNA export from the nucleus [106,111,112], possibly by binding and inhibiting components of the nuclear mRNA export machinery (Figure 1) [113]. The relative contributions to IFN inhibition of NS1 specifically targeting the RIG-I pathway versus its ability to limit general gene expression have not yet been addressed, and may vary between different virus strains. Furthermore, blocking general gene expression may limit other non-IFN-related pathways that also benefit virus replication. For example, very recent studies have specifically implicated the 1918 pandemic influenza A virus NS1 protein in blocking expression of genes related to lipid metabolism [114], although the functional consequences of this have yet to be established.

The heterotrimeric viral polymerase complex, polymerase subunit basic (PB)1–PB2–polymerase subunit acidic (PA), mediates a cellular mRNA cap-snatching activity that is essential for priming transcription of viral mRNAs [115]. Inevitably, this will reduce the levels of capped host mRNAs that are translated into functional proteins and may, therefore, constitute an additional mechanism by which influenza viruses attenuate host cell gene expression, including that of *IFN-β* (Figure 1) [56]. Mechanistically, the PB1 protein indirectly participates in cap-snatching activity, in that once bound to the viral template it activates the cap-binding

activity of the PB2 subunit [116,117]. The PA subunit has recently been shown to encode the endonuclease activity that removes the cap from the host mRNA [118,119]. There may be some interplay between the ability of the viral polymerase to shut down host-cell protein synthesis and the ability of NS1 to limit IFN induction by binding CPSF30, as all these virus and host components have recently been detected in the same complexes [120].

Influenza viruses limit PAMP availability

As briefly discussed above, the byproducts of virus replication can be RNA PAMPs that activate the host innate IFN response. Although clearly also required for basic replication, the encapsidation of viral RNA into RNPs may be considered an additional mechanism by which influenza viruses limit the access of RIG-I to certain PAMPs. In particular, RNA encapsidation by nucleocapsid protein (NP) may limit the formation of dsRNA PAMPs by preventing annealing of negative- and positive-sense viral RNAs. Furthermore, given that PAMP sensors for RNA viruses have only been identified in the cytoplasm [13], the nuclear replication strategy of influenza viruses probably reduces the likelihood of PAMP detection (Figure 1).

Influenza viruses limit the antiviral effects of IFN

Several studies suggest that in addition to limiting IFN production, influenza viruses are also capable of downregulating signaling from type I and type II IFN receptors. First, influenza viruses have been reported to block signaling from the IFN- γ receptor by reducing the phosphorylation of STAT1 and its subsequent nuclear accumulation [121]. In addition, influenza virus infection induces the expression of suppressor of cytokine signaling (SOCS) proteins, which inhibit signaling from the IFN- α/β R (Figure 2) [122,123]. It is currently unclear whether influenza viruses actively suppress these IFN signaling pathways, or if these responses are part of the normal regulatory feedback mechanisms of the cell. Furthermore, given that the antiviral effects of IFNs require transcriptional upregulation of target genes, the ability of influenza viruses to efficiently shut down host cell protein synthesis (either via NS1-mediated [104] or cap-snatching mechanisms [115]) is also likely to dampen the IFN response. The apparent capacity of some influenza viruses to block IFN signaling at multiple levels may mean that these viruses are particularly efficient at preventing the establishment of an antiviral state within cells [103]. In this regard, it is noteworthy that a naturally occurring polymorphism (D92E) has been reported in NS1 that enhances its ability to limit the IFN-induced antiviral state, and this amino acid change is associated with influenza A virus virulence [124].

Influenza viruses limit the effects of IFN-inducible antiviral effectors

Surprisingly little is known about the roles of specific antiviral effector proteins in downregulating influenza virus replication. Remarkably, for those that have been partially characterized (such as PKR, OAS, ISG15 and MxA), it is clear that influenza viruses have evolved strategies to modulate their own sensitivity to effector-mediated inhibition. Influenza A viruses use two mechanisms to counteract the powerful antiviral activity of PKR [125]. First, influenza A virus infection upregulates the activity of the cellular inhibitor of PKR, p58^{IPK} (Figure 2) [126]. The mechanism by which this upregulation occurs is not entirely clear, although there is no increase in the total amount of p58^{IPK} protein [127]. Rather, evidence suggests that influenza A virus infection causes the dissociation of p58^{IPK} from its natural cellular inhibitor, heat shock protein 40 [128,129]. The influenza A virus NS1 protein has also been reported to bind directly to PKR in order to inhibit the conformational changes that regulate its activity [130]. Similarly, the influenza B virus NS1 protein is able to limit PKR activity, but this seems to be mediated by a distinct mechanism involving a dsRNA bridge between NS1 and PKR [131,132]. A key additional function of dsRNA binding by the influenza A virus NS1 protein appears to be the inhibition of OAS, possibly by sequestration of viral

dsRNA [130]. Whether the influenza B virus NS1 protein also counters OAS by dsRNA sequestration is unknown.

One apparently unique function of the influenza B virus NS1 protein is its ability to bind ISG15 and subsequently inhibit the IFN-stimulated conjugation of ISG15 to cellular proteins [133]. This property is not shared by the influenza A virus NS1 protein. The mechanism by which influenza B virus NS1 inhibits ISG15 conjugation is far from fully established, but is likely to rely on the disruption of key interactions between ISG15 and the cellular E1/E2/E3 activation and ligation machinery [133,134].

It is probable that most (if not all) antiviral effectors activated by influenza virus infections are to some degree circumvented by a virus strategy. The extent to which a particular virus is able to achieve this can vary from one strain to another, a factor that can be owing to species-specific adaptations and which may ultimately contribute towards virulence. For example, the highly pathogenic 1918 influenza A virus RNP complex appears completely insensitive to the antiviral effects of human MxA, whilst more contemporary nonpathogenic human viruses are mildly sensitive to MxA [135]. Intriguingly, many avian influenza A viruses seem highly susceptible to the antiviral actions of human MxA [135]. A similar strategy has been described whereby a highly virulent influenza A virus strain can effectively 'out-run' the antiviral restrictions mediated by Mx proteins, apparently by simply replicating faster than other low-pathogenic, Mx-sensitive virus strains [136,137].

Influenza viruses modulate cell death

In cells and tissues in which influenza viruses replicate, the induction of apoptotic cell death may be considered an antiviral mechanism by which the host attempts to limit viral spread. As such, influenza viruses may indirectly evade the antiviral effects of apoptosis by replicating at faster speeds (Figure 2) [138]. There have also been reports that the influenza A virus NS1 protein can inhibit apoptosis in infected cells [139–141]. This can be attributed, at least in part, to its ability to suppress both IFN induction and the antiviral effects of IFN [140]. Furthermore, evidence suggests that activation of PI3K signaling by NS1 may directly contribute to suppression of host cell apoptosis (Figure 2) [139,141–143]. The NS1 protein of influenza B viruses does not bind or activate PI3K [144], and the role of this NS1 protein in limiting apoptosis during infection has yet to be firmly established.

Rather than blocking apoptosis, several studies have concluded that influenza viruses actually enhance cell death for a virus-supportive function. It is conceivable that by killing infected cells, virus release is enhanced. In addition, stimulating apoptosis may reduce cell-mediated cytotoxic responses, as infected cells are cleared by phagocytosis. A recent study has proposed that influenza A viruses target and destroy natural killer cells, thereby reducing the pool of innate immune cells that normally help to clear virus infections [145]. The mechanism of this immune cell killing is unknown, but could be due in part to expression of the viral PB1 frame 2 (PB1-F2) protein. PB1-F2 is a small 87-amino acid protein encoded by an alternate (+1) reading frame within the *PB1* gene [146]. PB1-F2 localizes to mitochondrial membranes [146–148], and its expression induces the formation of nonspecific pores within membranes [149]. PB1-F2 interacts with the mitochondrial apoptotic mediators adenine nucleotide translocator 3 and voltage-dependent anion channel 1 [150], thereby sensitizing cells to apoptotic cell death [146,147,149–151]. The proapoptotic effect of PB1-F2 is most pronounced in 'immune' monocyte/macrophage cells [146], and data from both reassortant and mutant viruses indicate that PB1-F2 contributes significantly towards viral pathogenesis [152–154]. Mechanistically, it has therefore been suggested that the proapoptotic function of PB1-F2 serves to limit efficient immune-cell-mediated virus clearance *in vivo* [152]. It should be noted that other influenza virus proteins have been linked to induction of apoptosis (e.g., NA [155, 156] and matrix protein 1 [M1] [157]), but the biological reasons for these functions remain

unclear. Interestingly, a connection between influenza A virus-induced apoptosis and perturbation of autophagosome maturation by the viral M2 ion channel has recently been established [158]. However, how regulation of autophagosomes contributes to influenza A virus replication and virulence remains to be determined [158,159].

Future vaccines & antivirals

Novel approach to generating live-attenuated influenza virus vaccines

As outlined above, influenza viruses are poor inducers of IFN, a property mostly attributable to the IFN-antagonistic functions of NS1. Thus, recombinant influenza viruses that express truncated forms of the NS1 protein (or full-length NS1 with particular amino acid substitutions in known functional regions) are attenuated for replication in cell culture and animal models [38,78–80,83–86]. Furthermore, in addition to perturbation of innate immune responses, the influenza virus NS1 protein can limit adaptive immune responses [160]. For example, whereas infection of myeloid-derived dendritic cells and primary human lung epithelial cells with wild-type influenza virus results in poor IFN production, infection with a recombinant influenza virus lacking the *NS1* gene induces robust IFN production [15,16]. Dendritic-cell maturation, which is required for priming of T cells, can also be stimulated by autocrine/paracrine IFN signaling [20,161,162]; however, the ability of NS1 to block IFN production by dendritic cells probably suppresses this mechanism of dendritic cell maturation during influenza virus infections. In addition, direct inhibition of proinflammatory transcriptional pathways by NS1 (such as RIG-I-mediated activation of NF- κ B) may also contribute to reduced dendritic cell maturation [163]. Indeed, a recombinant influenza virus expressing a truncated form of the NS1 protein was shown to be defective in blocking dendritic cell maturation and priming of T cells [16].

Increased immunogenicity of influenza viruses expressing truncated NS1 proteins has been demonstrated in mice and chickens [76,80,82]. Based on these observations, influenza viruses expressing truncated NS1 proteins are being developed as live-attenuated vaccines. Indeed, the vaccine potential of recombinant influenza viruses expressing mutant forms of NS1 has been proven in mice, chickens, swine, horses and macaques [76,80,81,84–86,164]. This novel approach to generating live-attenuated influenza virus vaccines has also been applied with similar success to influenza B viruses [165,166]. The benefit of using a virus expressing a truncated NS1 (rather than a full deletion) is that the virus can still replicate to some extent in the inoculated subject. Thus, lower doses of vaccine are required, and the vaccine can be administered intranasally (a factor that should allow development of mucosal immunity, which may be particularly important for host responses against respiratory viruses). Despite this novel strategy for generating the ‘backbone’ of live-attenuated viruses, both seasonal and pandemic vaccines would still require incorporation of viral antigens (usually hemagglutinin and NA) specific to the circulating virus.

The need for new antivirals

Protective vaccination is without doubt the best means of controlling both seasonal and pandemic influenza virus outbreaks. However, it can take a significant amount of time to generate an effective prophylactic vaccine, which has to be based on the specific antigenic nature of any emerging virus. Effective antiviral drugs are therefore critically important as short-term therapies in the event of outbreaks of a novel influenza virus to which a susceptible population has little, or no, prior immunity (e.g., as seen with the recent emergence of the novel swine-origin pandemic H1N1 influenza A virus). In this regard, antivirals should ‘buy time’ for specific vaccine development, manufacture and distribution.

There are currently only two classes of antiviral compound licensed by the US FDA that are available for the treatment of influenza viruses. Adamantanes (amantadine and rimantadine) act by blocking activity of the viral M2 proton channel, thus preventing virus uncoating and inhibiting release of the viral genome into host cells [1]. The neuraminidase inhibitors (oseltamivir and zanamivir) bind and block enzymatic activity of the viral NA protein [1]. This prevents efficient release of mature virions from the surface of infected cells and thereby limits viral spread (Table 1). Unfortunately, there is emerging national and global resistance to oseltamivir amongst circulating seasonal H1N1 influenza A viruses (>99%), although most H1N1 viruses remain sensitive to zanamivir [167]. Such extensive oseltamivir resistance has not been observed for either H3N2 influenza A viruses or influenza B viruses. All currently circulating H3N2 influenza A viruses are adamantane resistant, and this drug is not effective against influenza B viruses [167]. Thus, current effective antiviral therapy requires either precise virus subtyping or a combination of drugs to be administered. The occasional observation of oseltamivir resistance among highly pathogenic H5N1 viruses would be another reason to develop novel antivirals in case these viruses prove successful in establishing a new devastating pandemic [168,169]. Although early isolates of the novel swine-origin pandemic H1N1 influenza A virus were demonstrated to be susceptible to the commonly used antivirals [170], some oseltamivir-resistant mutants of this virus have recently been reported [171]. Thus, it is evident that new antivirals are continually (and perhaps urgently) needed. Although a number of studies have proposed multiple excellent strategies for the future development of novel anti-influenza drugs [172–174], we focus here only on the potential to target specifically the interaction of influenza viruses with host innate immune responses. Other current and potential targets are summarized in Table 1.

NS1 protein as an antiviral target

Although apparently nonessential for influenza virus replication [38,88,175], the NS1 protein is emerging as an attractive antiviral target. Multiple studies have demonstrated that influenza viruses engineered to express NS1 proteins lacking specific functions (e.g., dsRNA binding, CPSF30 binding, PI3K binding or TRIM25 binding) are attenuated in tissue culture systems and/or animal models [83,97,104,109,132,142,176,177]. Thus, one future anti-influenza strategy may be to target these conserved interactions with small molecule inhibitors. Indeed, stable expression of a ‘competitive’ CPSF30 fragment in a tissue culture cell line prevents NS1 from binding and inhibiting endogenous CPSF30 during infection, and limits efficient influenza A virus replication without affecting normal cellular mRNA processing [105]. Other studies beyond this first ‘proof-of-principle’ approach have further validated the rationale that targeting functions of NS1 may lead to effective future therapeutics. Basu *et al.* recently developed a yeast-based high-throughput assay to screen for chemical inhibitors of NS1-mediated growth inhibition [178]. As a result, four small molecular weight compounds from the existing NIH National Cancer Institute Developmental Therapeutics Program (NSC125044, NSC128164, NSC109834 and NSC95676) were identified that specifically counteract the ability of different NS1 proteins to limit IFN- β induction [178]. Furthermore, these inhibitors could reduce influenza A virus (but not respiratory syncytial virus) replication in tissue culture [178]. Although the precise mechanisms of action of these compounds remain to be elucidated, these results should encourage other researchers to pursue chemical compound and natural product library screening with the aim of developing defined inhibitors of NS1 function.

The recent crystallization and structure determination of a full-length NS1 protein [179], specific NS1 domains [180–184] and NS1 in complex with either dsRNA [185] or a fragment of CPSF30 [109], have provided an excellent initial framework for future structure-based rational drug design (reviewed in [172]). Of particular interest is the RNA-binding domain of NS1, which is highly conserved structurally between both type A and type B influenza viruses

[184]. For influenza A viruses this domain of NS1 contributes to at least two important functions: inhibition of 2'–5' OAS/RNase L [176], and the inhibition of TRIM25/RIG-I-mediated IFN production [42,97]. For influenza B viruses, the NS1 RNA-binding domain is also implicated in the inhibition of IFN production [89], as well as limiting PKR activity [132]. Thus, given that the mechanism of dsRNA binding by these two NS1 proteins is likely to be similar (reviewed in [172]), it has been postulated that a deep pocket on NS1 at its interface with dsRNA could be targeted with small-molecule inhibitors [184]. Indeed, as the NS1 RNA-binding domain protein fold appears to be unique (i.e., not found in any cellular proteins to date [182,186]), compounds designed to bind this site may exhibit high specificity and a reduced likelihood of off-target side effects. A focused *in vitro* high-throughput screening assay has recently been successful in identifying three low molecular-weight compounds with drug-like properties that target the RNA-binding activity of the influenza A virus NS1 protein [187]. It will be of great interest to determine the crystal structures of such lead compounds in complex with NS1 (possibly of both influenza type A and B viruses). Subsequent *in vitro* structure-based chemical optimization may yield more potent and specific inhibitors that can be used for further antiviral evaluation, both in tissue culture and suitable animal models.

The crystal structure of the NS1–CPSF30-binding interface also reveals a promising rational target for the development of antivirals specific to influenza A viruses [109]. The major site on NS1 that interacts with CPSF30 is a conserved hydrophobic pocket formed between the long α -helix and second β -strand of the NS1 effector domain (ED). During binding, several aromatic residues of CPSF30 appear to dock into this site (Figure 3a) [109]. Remarkably, this same NS1 hydrophobic pocket is essential for the inherent homodimerisation capability of the NS1 ED, whereby the tryptophan-187 residues of two ED monomers reciprocally dock into the hydrophobic pockets of the neighboring ED monomer (Figure 3B) [181,183]. Clearly, this implies that CPSF30 binding and (at least) one NS1 ED multimerization form are mutually exclusive, a concept likely to be fundamentally important in trying to understand the multifunctional nature of NS1 [75]. In addition, this observation suggests that antivirals targeting this conserved hydrophobic pocket on NS1 could block at least two separate NS1 processes: CPSF30-binding and a structural conformation of NS1 likely required for a different function. As outlined earlier, one caveat with designing inhibitors of viral proteins is always the potential for resistance mutations to arise. However, targeting a single region on NS1 that has two distinct functions may mean resistance mutations are less likely to occur.

Targeting host cell innate immune pathways as an antiviral strategy

Targeting host factors with antivirals is an additional way to avoid the development of drug resistance. Such a strategy is unlikely to be virus strain specific, in that any new antiviral may be effective against multiple influenza virus subtypes. Thus, the systematic identification of cellular proteins required for influenza virus replication could yield potential new druggable candidates [188,189]. In addition, compounds that stimulate or augment the host antiviral response may rapidly dampen down virus replication, even in the face of strong viral innate immune evasion mechanisms. The administration of exogenous recombinant IFN- α has been reported to be an effective prophylactic therapy against influenza viruses in mice [190], guinea pigs [37,191] and ferrets [41]. As with many drugs targeting host pathways, there is the potential for toxic side effects to occur caused by the deregulation of cell signaling pathways in uninfected tissues. Indeed, side effects associated with repeated intranasal administration of IFN- α may include nasal mucosal irritation and occasional bleeding (reviewed in [41]). However, it is likely that such side effects may be tolerable in the event of a serious pandemic outbreak, particularly if the therapy is only used in the short term. Clearly, further *in vivo* and clinical re-evaluation of this antiviral strategy is warranted, and it may be that other IFN agonists are more suitable (for recent examples see [192,193]), particularly if novel compounds can be designed that exhibit less toxicity than IFN itself. However, the immediate advantage

of adopting IFN as a potential anti-influenza therapy is that preparations are already approved for human use in the treatment of chronic HBV and HCV infections (IFN- α), and multiple sclerosis (IFN- β) [194].

Future perspective

The currently circulating swine-origin H1N1 pandemic influenza A virus provided somewhat of a surprise to the research community, who were focused on highly pathogenic avian viruses in poultry. This new virus appears to have already caused significant morbidity and mortality worldwide, and is clearly an ongoing public health concern that will occupy our focus for the near future. The immediate priority must be to monitor the evolution of this pandemic virus in order to assess whether it acquires known virulence determinants. Of course, the development of effective countermeasures (such as protective vaccines) to combat the consequences of human infection is essential. The technical abilities that we have as a scientific community, combined with the rapid response of many laboratories, means that now more than ever we are well positioned to deal effectively with this virus.

From a purely research point of view, the swine-origin H1N1 virus is likely to provide interesting insights into how pandemic influenza viruses are generated. Data from whole-genome sequencing efforts, together with 'real-time' *in vivo* and *in vitro* laboratory studies (which will be essential for monitoring any virulence changes in this virus) should give us a better understanding of how influenza viruses adapt to new species, and what the barriers to species-species 'jumps' are. Identification of the precursor to this emergent pandemic virus, which has presumably circulated in pigs for some time, will obviously be necessary.

We already have some idea that the host innate immune response can be a significant restriction to influenza viruses adapted to an alternative host [195]. Indeed, avian and human influenza viruses exhibit differential susceptibility to the IFN-inducible mouse Mx1 and human MxA antiviral proteins [135]. Furthermore, it is unclear why highly pathogenic H5N1 influenza viruses replicate poorly and cause mild disease in swine [196], yet are highly virulent in mice, chickens, ferrets and macaques. Similarly, the reconstructed 1918 pandemic influenza virus causes moderate pulmonary pathology in swine [197], but induces a fatal disease course in mice, ferrets and macaques [25,198,199]. Even more remarkable is the complete lack of virulence of both highly pathogenic H5N1 influenza viruses and the reconstructed 1918 pandemic influenza virus in guinea pigs, despite the high levels of virus replication in the respiratory tract [37]. Overall, this suggests that there is a major host component to pathogenicity. Thus, we still need to understand the molecular changes required by a virus to allow it to circumvent particular host-specific responses. Following on from this, most current research into innate immune processes and viral countermeasures is biased towards human model systems, and future work could also address how these virus-host interactions work in other species. From a practical point of view, this may be particularly important for animal vaccine development and antiviral drug design. From a comparative biology point of view, such studies will continue to help us uncover how normal cellular processes work.

Executive summary

Influenza viruses

- In humans, influenza virus infection is usually limited to the upper respiratory tract. In certain severe cases the lower respiratory tract can be affected, causing viral pneumonia often exacerbated by secondary bacterial infection.
- 'Seasonal influenza' is caused annually by new viruses possessing slightly different surface antigens. Such epidemics account for approximately 500,000 deaths worldwide.

- Global influenza pandemics occur when antigenically novel influenza viruses emerge. The most devastating to date, in 1918, was responsible for over 40 million deaths.
- In March 2009, a novel swine-origin H1N1 influenza A virus emerged from pigs into the human population and has spread to become the latest influenza pandemic.

Host innate immune response to infection

- The interferon (IFN) system is a powerful innate immune defence used by cells to limit virus replication.
- Viral pathogen-associated molecular patterns (PAMPs) are detected by host cell sensors (e.g., retinoic acid-inducible gene-I), which then initiate a complex signaling cascade resulting in the synthesis and secretion of IFNs.
- IFNs can act in either an autocrine or paracrine manner to upregulate over 300 antiviral genes that ultimately lead to suppression of virus propagation.

Influenza virus antagonism of innate immunity

- Influenza viruses have evolved numerous strategies in order to evade the host innate immune response.
- The viral nonstructural (NS)1 protein acts at multiple levels to prevent IFN production: NS1 can limit PAMP detection, block essential retinoic acid-inducible gene-I post-translational modifications and block the processing and export of IFN mRNAs.
- NS1 may also limit IFN responses by preventing expression of antiviral proteins or by directly inhibiting their activities.
- Other strategies used by influenza viruses to evade the effects of IFN include: limiting PAMP formation, general inhibition of host cell protein synthesis, replication compartment, replication speed, induction/inhibition of apoptosis and reduced sensitivity to host antiviral proteins.

Future vaccines & antivirals

- Vaccination is the best means of controlling both seasonal and pandemic influenza outbreaks. No 'universal' influenza vaccine exists; thus, new seasonal vaccines must be developed annually.
- One strategy for vaccine design is to use influenza viruses engineered to express mutant NS1 proteins. The replication of such viruses is restricted *in vivo*; thus, they are good candidates for live-attenuated vaccines.
- Antiviral drugs may be the 'first line of defence' whilst vaccines are generated to novel seasonal or pandemic viruses.
- The development of viral resistance to existing licensed antiviral drugs is a cause for concern; thus, new antivirals targeting other viral components are urgently required.
- Several sites on the influenza A virus NS1 protein may be targets for novel antivirals.
- IFN and other agonists of host innate immunity may be useful as future therapeutics.

For antivirals, it is clear that a number of alternative strategies are currently being pursued and are at various stages of development (reviewed in [172]). A molecular understanding of the structural mechanics of influenza virus replication (and not least the interaction of viral proteins with cellular factors) is likely to drive the discovery and validation of new antiviral targets. As soon as a library of these new drugs becomes licensed for use in humans, our ability to control outbreaks of potentially prepandemic influenza viruses will increase, particularly with the use of ever-changing combination therapies.

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Figure 1. Retinoic acid-inducible gene-I-mediated type I interferon pathway and its regulation during influenza A virus infection

(A–E) Critical checkpoints. (A) Virus replication produces triphosphorylated vRNA and potentially dsRNA byproducts that are pathogen-associated molecular patterns recognized by cytoplasmic RIG-I. (B) NS1 regulates the activation of RIG-I by binding to and sequestering dsRNA and/or by interaction with RIG-I. Formation of viral RNP complexes may also contribute to ‘hiding’ pathogen-associated molecular patterns from RIG-I. (C) Binding of NS1 to TRIM25 prevents essential ubiquitination of RIG-I. (D) Cap-snatching activity of the viral polymerase complex may reduce the pool of host antiviral mRNAs available for nuclear export and translation. The NS1 protein also directly inhibits global cellular pre-mRNA processing by binding to host CPSF30. (E) NS1 binds to components of the nuclear pore complex and inhibits nuclear export of cellular mRNA.

CPSF30: 30-kDa cleavage and polyadenylation specificity factor; IRF: Interferon regulatory factor; MAVS: Mitochondrial antiviral signaling adaptor; NEMO: NF-κB essential modulator;

NS1: Nonstructural protein 1; PPP: Triphosphate; RIG: Retinoic acid-inducible gene; RIP: Receptor-interacting protein; RNP: Ribonucleoprotein; TANK: TRAF family member-associated NF- κ B activator; TRAF: TNF-receptor-associated factor; TRIM: Tripartite motif; Ub: Ubiquitin.

Figure 2. Type I interferon receptor signaling pathway and expression of interferon-stimulated genes

Binding of IFN- α/β to the IFN receptor stimulates the phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and 2 associate with IRF-9 to form the transcription factor ISGF3, which relocates to the nucleus and stimulates the transcription of ISGs whose promoters contain IFN-stimulated response elements. Although IFN stimulates the transcription of more than 300 ISGs, only the antiviral functions of a small percentage have been well characterized. Notable examples of IFN-induced antiviral effectors and their regulation by influenza viruses are indicated. See text for further details.

A/NS1: Influenza A virus NS1 protein; B/NS1: Influenza B virus NS1 protein; IFN: Interferon; IRF: IFN regulatory factor; ISG: IFN-stimulated gene; MxA: Myxovirus resistance gene A; NP: Nucleocapsid protein; NS1: Nonstructural protein 1; OAS: 2'-5' oligoadenylate synthetase; SOCS: Suppressor of cytokine signaling.

Figure 3. Potential multifunctional antiviral target on the influenza A virus NS1 protein

(A) Surface representation of an NS1 ED monomer (dark gray) in complex with a fragment of CPSF30 (light gray, cartoon ribbon representation). Aromatic residues of CPSF30 that dock into the NS1 hydrophobic pocket [109] are highlighted as sticks. (B) Surface representation of NS1 ED in complex with a fragment of NS1, as seen in helix–helix dimeric conformations of NS1 [181,183]. Tryptophan-187 of the neighboring NS1 monomer docks into the same hydrophobic pocket as that required for CPSF30-binding. Images were prepared using MacPyMol (Protein Data Bank files: 2RHK & 3EE9).

CPSF30: 30-kDa cleavage and polyadenylation specificity factor; ED: Effector domain; NS1: Nonstructural protein 1.

Table 1
The 11 proteins encoded by influenza A viruses, their functions and potential antiviral susceptibility

Protein	Segment	kDa	Major Function(s)	Antiviral targets	Ref.
HA	4	64	Attachment to host cells; fusion of viral and host membranes	Fusion inhibitors (e.g., TBHQ*) Sialic acid removal (e.g., recombinant sialidase DAS181*)	[200] [201,202]
NA	6	50	Cleavage of sialic acid; release of virions from cells	Neuraminidase inhibitors (oseltamivir [‡] , zanamivir [‡] and CS-8958*)	[203]
PB1	2	87	Polymerase component	PB1-PA interaction Polymerase function (e.g., T-705*)	[204] [205]
PB2	1	86	Polymerase component	Cap binding	[116,172]
PA	3	83	Polymerase component	PB1-PA interaction Endonuclease	[204] [118,119]
NP	5	56	RNA encapsidation; RNP component	RNA binding? Oligomerization pocket?	[172]
M1	7	28	Matrix protein; structural support of virion	Oligomerization?	
M2	7 (spliced)	11	Ion channel; dissociation of viral components during uncoating	Ion channel inhibitors (amantadine [‡] and rimantidine [‡]).	
NS1	8	26	Antagonism of host immune responses	RNA binding, CPSF30 binding, IFN antagonism*	[187] [105] [178]
NEP	8 (spliced)	14	Nuclear export of RNPs	Sites of RNP interaction?	
PB1-F2	2 (+1 ORF)	8	Induction of apoptosis in immune cells	Sites of interaction with cellular proteins? Oligomerization?	

For each protein, its corresponding RNA genomic segment is indicated, as well as its approximate molecular weight (in kDa).

* Compounds identified with antiviral activity in experimental trials.

[‡] US FDA-approved antivirals.

CPSF30: 30-kDa cleavage and polyadenylation specificity factor; F2: Frame 2; HA: Hemagglutinin; IFN: Interferon; M1: Matrix protein 1; NA: Neuraminidase; NEP: Nuclear export protein; NP: Nucleocapsid protein; NS: Nonstructural; ORF: Open reading frame; PA: Polymerase subunit acidic; PB: Polymerase subunit basic; RNP: Ribonucleoprotein; TBHQ: Tert-butyl hydroquinone.