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Induction and evasion of type I interferon responses by influenza viruses

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Abstract

Influenza A and B viruses are a major cause of respiratory disease in humans. In addition, influenza A viruses continuously re-emerge from animal reservoirs into humans causing human pandemics every 10–50 years of unpredictable severity. Among the first lines of defense against influenza virus infection, the type I interferon (IFN) response plays a major role. In the last 10 years, there have been major advances in understanding how cells recognize being infected by influenza viruses, leading to secretion of type I IFN, and on the effector mechanisms by how IFN exerts its antiviral activity. In addition, we also now know that influenza virus uses multiple mechanisms to attenuate the type I IFN response, allowing for successful infection of their hosts. This review highlights some of these findings and illustrates future research avenues that might lead to new vaccines and antivirals based on the further understanding of the mechanisms of induction and evasion of type I IFN responses by influenza viruses.

1. Introduction

Influenza virus is an important human respiratory pathogen that causes annual epidemics and every 10–50 years, pandemics of different severity. In addition, influenza viruses circulate in other animals, including pigs, horses, dogs, poultry and waterfowl birds. Host tropism of influenza virus is determined by multifactorial viral genetic factors. Adaptation of an influenza virus strain circulating in an animal host to infect and propagate in humans constitutes the basis of pandemics. Innate immunity and specially interferon responses represent an early barrier that influenza virus, as well as any other virus, needs to break in order to be a successful pathogen. Mammalian cells have evolved sophisticated antiviral mechanisms based on sensing viral products and triggering of signaling cascades leading to secretion of type I interferon (IFN β and IFN α) and type III interferons (IFN λ), that inhibit viral replication and contribute to the initiation of more specific adaptive immune responses. This review summarizes the ways the host induces interferon in response to influenza virus infection, how interferon inhibits influenza virus replication, and how influenza virus successfully attenuates this host response to establish successful infections, with a focus on some of the most recent advances in our understanding of this complex set of virus-host

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interactions and their potential therapeutic implications. The impact that these interactions have in the ability of influenza viruses to adapt to new hosts and on disease severity is also discussed.

2. Induction of type I interferon responses by influenza viruses

The ability of cells to recognize products derived from viruses constitute the basis for the initiation of the antiviral response. How cells can recognize self-products from viral products that are made of cellular components? This apparent impenetrable problem has been solved through the use of cellular sensors that recognize specific nucleic acids in unusual locations which are associated with ongoing viral infections. Two major types of sensors have been shown to be involved in induction of interferon during influenza virus infection: TLRs and RLRs (Fig. 1).

2.1. TLRs

Toll-like receptors (TLRs) were the first receptors that were recognized as able to sense viral nucleic acids (Kawai and Akira, 2011). Especially important for RNA viruses, such as influenza virus, are TLR3, that recognizes dsRNA, and TLR7/8 that recognize ssRNA (Blasius and Beutler, 2010). Although such molecules are not specific for viruses, the presence of RNA in the endosome, which is where these TLRs are located, is in general associated with RNA virus infections. All TLRs are transmembrane proteins whose extracellular domain is implicated in the recognition of molecular signatures associated with pathogens. Influenza virus, as well as many other RNA viruses, enters the endosomal compartment as part of its replication cycle: upon binding to its receptor, influenza virions are internalized into endosomes where acidification of the pH triggers the fusion event required for viral entry into the cytoplasm. In addition, the virus can also be internalized into endosomes by specialized cells that phagocytose antigens and pathogens, such as macrophages, dendritic cells (DC) and B cells. As viral escape from the endosome is not a 100% efficient process, some virions are likely degraded in the endosome, exposing the viral RNA that is now recognized by TLR3 (due to the presence of dsRNA areas in the genomic RNA segments) and TLR7/8 (Lund et al., 2004). In addition, viral RNA can also access the endosomal compartment from the cytoplasm of infected cells by autophagic processes (Lee et al., 2007) and by secreted exosomes and/or other cell to cell transfer mechanisms that results in viral RNA internalization by adjacent cells (Takahashi et al., 2010). Binding of RNA to TLRs results in the recruitment of adaptor molecules through the TIR domains located in the TLR cytoplasmic tails, TRIF in the case of TLR3 and Myd88 in the case of TLR7/8 (Kawai and Akira, 2011). Activation of TLR3/TRIF in macrophages and DCs results in IRF3 and NF- κ B activation, and these transcription factors cooperate in the induction of IFN β . Activation of TLR7/MyD88 in plasmacytoid DCs, believed to be cell specialized in secretion of IFN, results in IRF7 and NF- κ B activation, and in secretion of high levels of IFN β and IFN α . While it is well established that influenza virus activates TLR3 and TLR7 pathways (Le Goffic et al., 2007; Lund et al., 2004), this activation alone does not explain the IFN-mediated antiviral response during infection. Mice deficient in TLR3 are not more susceptible to influenza virus infection, and if anything, they develop less severe disease, most likely associated with lower induction of pro-inflammatory cytokines (Le Goffic et al., 2006). The presence of TLR7/8 in highly specialized cells cannot account for the known induction of IFN in infected epithelial cells, the main target of influenza virus infection.

2.2. RIG-I

In 2004 Yoneyama et al (Yoneyama et al., 2004) identified the cellular sensor that accounts for induction of IFN in influenza virus infected epithelial cells, the Retinoic Inducible Gene

I or RIG-I. RIG-I, and the other two members of what is known now as the family of RIG-I Like Receptors (RLRs), MDA-5 and LGP2, sense viral RNA products in the cytoplasm of infected cells. Sensing viral RNA products in the cytoplasm represents a bigger challenge than sensing these products in endocytic compartments. Thus, while the presence of RNA in endocytic compartments is believed to be an unusual event associated with viral infection, the cytoplasm is full of many different types of cellular RNAs, such as mRNA, tRNA, rRNA, microRNAs and other small RNAs. The RLRs are highly sophisticated molecular sensors that have been specialized in distinguishing viral RNA from the rest of cellular RNAs (Loo and Gale, 2011). The best characterized RLR to date is RIG-I, which is also known to be the main sensor of influenza virus infection leading to IFN induction (Kato et al., 2006). Thus, cells in which RIG-I has been suppressed by knocking the gene out or by silencing are unable to produce IFN in response to influenza virus infection. A critical molecular motif that is exploited by RIG-I to distinguish viral RNA from cellular RNA is the presence of a 5'-triphosphate (Hornung et al., 2006; Pichlmair et al., 2006). This is a common feature of the RNA genomes of most negative strand RNA viruses, including influenza virus. The 5' ends of the majority of the cellular cytoplasmic RNAs is being processed in the nucleus resulting in the elimination or on the masking of the 5'-triphosphate. 5'-triphosphate containing RNA is being recognized by the carboxy-terminal domain of RIG-I, also known as the repressor domain (RD) (Cui et al., 2008; Lu et al., 2010; Takahashi et al., 2008; Wang et al., 2010). In addition to the RD, RIG-I contains two Caspase Associated Recruitment Domains (CARD) at the N-terminal, and an RNA helicase domain in the middle of the molecule. In the inactive state, the RD domain holds the CARD domains in an inactive conformation (Saito et al., 2007). Upon binding to the RD to viral RNA, the helicase domain of RIG-I becomes activated, and the molecule translocates in the RNA while hydrolyzing ATP (Myong et al., 2009). This process results in a conformational change that exposes the CARD domains, which become ubiquitinated by the action of E3 ligases such as TRIM25 (Gack et al., 2007) and RIPLET (Oshiumi et al., 2010). Ubiquitin then promotes the interaction of RIG-I with the downstream adaptor MAVS and subsequent activation of IRF-3 and NF- κ B, leading to type I IFN production. Since the discovery of RIG-I, there has been a great interest in identifying the nature of the RNA ligands that can activate this sensor. Studies with exogenously introduced RNAs into RIG-I containing cells revealed that the structure, length, sequence composition and presence of 5'-triphosphate are all features that influence the ability of a given RNA to be recognized by RIG-I (Hornung et al., 2006; Kato et al., 2008; Malathi et al., 2007; Marques et al., 2006; Pichlmair et al., 2006; Ranjith-Kumar et al., 2009; Saito et al., 2008; Schlee et al., 2009; Schmidt et al., 2009). But only recently the RNA bound to RIG-I during influenza virus infection has been analyzed. Using primer extension and Northern blots, Rehwinkel et al (Rehwinkel et al., 2010) found genomic influenza virus RNA bound to RIG-I during infection after RIG-I overexpression and immunoprecipitation. Similarly, unbiased deep sequencing of RNA bound to immunoprecipitated endogenous RIG-I during influenza virus infection revealed the presence of genomic viral RNA, with a marked preference for the shorter influenza virus segments, including internally deleted defective interfering viral RNAs (Baum et al., 2010). The presence of a 5'-triphosphate in the viral RNA was essential for RIG-I activation. Interestingly, influenza viral RNA is characterized by the presence of partial dsRNA structures formed by the 5' end 3' ends, which may also contribute to optimal RIG-I recognition. The molecular reason for the size preference of the RNAs recognized by RIG-I is still unknown, but the recently determined structures of different RIG-I domains will help in the near future to better understand the mechanism of activation of this cellular sensor (Civril et al., 2011; Jiang et al., 2011; Kowalinski et al., 2011; Luo et al., 2011).

3. Inhibition of influenza virus by type I interferon

Type I IFN induces the JAK/STAT pathway upon binding to its receptor, IFNAR, and this induction results in the transcriptional stimulation of interferon stimulated genes, or ISGs, which in turn restrict viral replication. There are more than 300 genes induced by IFN, and not all of them have been characterized with respect to their antiviral activity (Der et al., 1998). We will summarize the ability of some of these ISGs to restrict influenza virus replication.

3.1. Mx

The orthomyxovirus resistance gene, or *Mx*, was the first ISG found to restrict influenza virus replication (Haller et al., 1980). The product of this gene, Mx1, was identified as responsible for resistance to influenza virus infection in mice. Most of the laboratory strains of mice lack a functional Mx1 and therefore are highly susceptible to influenza virus infection (Staeheli et al., 1988). Both mouse Mx1 and the human homologue, MxA, inhibit influenza virus replication. MxA is a GTPase with close homology to the dynamin GTPases, and inhibits a large variety of viruses, including influenza (Haller et al., 2009). Although its antiviral mechanism of action is not fully elucidated, MxA appears to oligomerize to form rings around the ribonucleocapsids of influenza and other viruses, then blocking viral replication (von der Malsburg et al., 2011). Recently, the structure of MxA has been solved, which revealed intra- and intermolecular interactions required for their antiviral activity, consistent with the proposed ring model of inhibition of viral replication (Gao et al., 2011). However, more research is required to understand the broad specificity of MxA in inhibiting multiple viruses with no apparent sequence identities in their ribonucleoproteins.

3.2. PKR

PKR is an IFN inducible protein kinase that becomes activated upon binding to dsRNA. While there have been multiple substrates identified for PKR, most of the antiviral activity of PKR is due to phosphorylation of eIF2 α , which results in a general translational block, limiting viral replication (Pindel and Sadler, 2011). The importance of PKR in restricting influenza virus replication has been best illustrated by studies in PKR knock-out mice. These mice are highly susceptible to infection with NS1 defective influenza virus strains (Bergmann et al., 2000). As discussed below, the influenza virus NS1 inhibits the induction of IFN as well as the activity of PKR, and therefore, the impact of PKR in influenza virus infection is best evidenced in the absence of NS1 function.

3.3. OAS

OAS, or oligoadenylate synthetase, is the first component of the OAS/RNaseL antiviral system. Like PKR, OAS is IFN inducible but requires binding to dsRNA for activation of its enzymatic activity. Once activated, OAS generated 2'-5' oligoadenylates that act as a co-factor for a latent cytoplasmic RNase, RNaseL. Activated RNaseL cleaves viral and cellular RNA stopping viral replication (Chakrabarti et al., 2011). In addition, it has also been shown that the cleaved products of RNaseL can activate RIG-I, and therefore serve as a positive feed-back to enhance IFN production (Malathi et al., 2007). Treatment with IFN is less effective in inhibiting influenza virus replication in RNaseL silenced cells, demonstrating the importance of the OAS/RNaseL system in restricting viral replication (Min and Krug, 2006).

3.4. ISG15

ISG15 is a small protein which structurally resembles two covalently linked ubiquitins (Narasimhan et al., 2005). Like ubiquitin, ISG15 is conjugated into proteins through Lysine

residues. While the consequences of ISGylation are still unclear, ISG15 knock-out mice are more susceptible to infection by several viruses, including influenza A and B viruses, demonstrating the antiviral activity of this molecule (Lenschow et al., 2007). Importantly, mice lacking the ISG15 E1 enzyme UBE1L required for ISG15 conjugation are also more susceptible to influenza virus infection, indicating that ISG15 exerts its antiviral activity by conjugation (Lai et al., 2009). One of the influenza viral proteins that have been shown to be conjugated by ISG15 is the NS1 protein, and this conjugation inhibits NS1 function (Tang et al., 2010; Zhao et al., 2010), but whether this is the main ISGylation event that inhibits influenza virus replication is unclear.

For instance, multiple cellular proteins are also ISGylated upon IFN treatment, especially those undergoing active translation (Durfee et al., 2010), and ISGylation of cellular proteins may also contribute to the antiviral activity of ISG15. The ability of ISG15 to restrict influenza virus replication has also been demonstrated in human cells infected with influenza viruses (Hsiang et al., 2009).

3.5. Viperin, tetherin and IFITMs

Some of the more recently studied ISGs with respect to their ability to inhibit influenza virus infection are viperin, tetherin and the IFITMs. Viperin is an IFN inducible protein that localizes in the ER, where it was found to interact with and inhibit farnesyl diphosphate synthase, an enzyme required for isoprenoid biosynthesis. This inhibition leads to changes in membrane fluidity and membrane microdomains, which in turn affect the ability of influenza virus to efficiently bud from infected cells, then restricting viral replication (Wang et al., 2007). Tetherin (also known as BST2) is like viperin an IFN inducible protein that restricts viral budding. In the case of tetherin, this is achieved by retaining newly assembled virions attached to the plasma membrane, as first demonstrated for retroviruses (Neil et al., 2008; Van Damme et al., 2008). While tetherin restricts formation of influenza virus like particles (Yondola et al., 2011), the impact of tetherin during influenza virus infection appears to be minimal (Watanabe et al., 2011). More research will be needed to understand the molecular reasons for these different observations. In contrast to the budding inhibitors viperin and tetherin, IFITMs are interferon inducible genes that restrict viral entry (Brass et al., 2009). While their precise mechanism of action is unclear, they seem to be specific inhibitors of viruses that enter through the endosomal pathway, including influenza viruses, suggesting that they affect the function of viral proteins involved in viral fusion in the endosome, i.e. in the case of influenza virus, the fusion activity of its HA protein (Huang et al., 2011).

4. Evasion of type I interferon responses by influenza viruses

While the IFN system has potent influenza virus antiviral activity, it is nevertheless unable to prevent influenza virus infections. This is due to the fact that influenza virus, as also many other successful viruses, have evolved strategies to attenuate the IFN response to levels that open a window for replication and transmission within their hosts. Interestingly, influenza virus uses multiple mechanisms to evade and inhibit the IFN response.

4.1. The NS1 protein

The non-structural protein of influenza virus NS1 has evolved multiple mechanisms to attenuate the IFN response during influenza virus infection, and it appears to be the most important IFN-antagonist protein encoded by the virus. This protein is highly expressed in the cytoplasm and the nucleus of infected cells where it is able to interact with different components involved in the IFN response, inhibiting this response (Hale et al., 2010a). First, the NS1 has an RNA binding domain located within its first 73 amino-terminal amino acids

that preferentially binds dsRNA (Liu et al., 1997). As dsRNA molecules are known activators of RLR sensors and of PKR and OAS, the NS1 competes with these cellular proteins for RNA binding, reducing their activation. Among RIG-I, PKR and OAS, binding of NS1 to dsRNA best inhibits OAS activation, as OAS has a lower affinity for dsRNA as compared to PKR and RIG-I (Min and Krug, 2006). Interestingly, the NS1 still efficiently inhibits PKR and RIG-I activation by interacting directly with PKR (Li et al., 2006) and by binding and inhibition the activity of TRIM25 (Gack et al., 2009), an E3 ligase required for optimal activation of RIG-I leading to IFN induction (Gack et al., 2007). Second, the NS1 inhibits in the nucleus the processing of cellular mRNAs inhibiting cellular gene expression. This inhibition is conducted by binding to CPSF (Nemeroff et al., 1998), a cellular factor involved in transcriptional termination and polyadenylation, and by binding to splicing (Fortes et al., 1994; Qiu et al., 1995) and nuclear export factors (Satterly et al., 2007), inhibiting the export of cellular mRNAs to the cytoplasm. The general inhibition of gene expression by the NS1 not only prevents efficient IFN expression but also expression of ISGs. The structure of the NS1 bound to RNA and to CPSF30 is now available, and amino acids in NS1 making contact with this factor have been mutated, resulting in recombinant viruses impaired in their ability to overcome the induction of IFN (Das et al., 2008). However, inhibition of CPSF30 is not fully conserved among influenza virus strains. For example, the new pandemic H1N1 expresses an NS1 protein that does not bind CPSF30. Despite that, this virus was highly successful infecting and transmitting within humans. Notably, mutations associated with gain of binding of NS1 to CPSF30 did not increase replication or pathogenesis in mice (Hale et al., 2010b). Thus, additional mechanisms might exist in some influenza virus strains to compensate for the lack of specific NS1 functions. In addition to inhibiting the IFN response, NS1 performs several other functions during infection, and it appears to exist in different dimeric conformations in the cell, consistent with its multifunctional character (Kerry et al., 2011). How these multiple functions of the NS1 are regulated and spatio-temporally controlled is still unclear, and is likely to be an intriguing area of research in the near future.

The NS1 protein of influenza B virus deserves special mention. Like the NS1 of influenza A virus, this protein has an N-terminal dsRNA binding domain implicated in inhibition of IFN induction and of PKR. However, it does not bind to CPSF and does not inhibit general gene expression. In contrast, the NS1 of influenza B virus, and not its influenza A virus counterpart, binds to ISG15 and directly inhibits ISGylation (Guan et al., 2011; Li et al., 2011; Yuan and Krug, 2001). This property is highly specific for ISG15 derived from specific host species, such as humans, which may in part explain the restricted host tropism of influenza B viruses (Sridharan et al., 2010; Versteeg et al., 2010).

4.2. The PB1-F2 protein

Some strains of influenza virus contain a second open reading frame within the PB1 gene that encodes a polypeptide of around 80 amino acids (Chen et al., 2001). This polypeptide, or PB1-F2 has been implicated in the virulence of the virus, with specific polymorphisms, such as N66S, found in the 1918 human pandemic strain of influenza virus, associated with increased pathogenesis (Conenello et al., 2007; McAuley et al., 2010; McAuley et al., 2007; Schmolke et al., 2011). Interestingly, PB1-F2 has a mitochondrial localization signal, and in the mitochondria appears to inhibit IFN induction most likely by interacting with MAVS, a critical mitochondrial adaptor required for IFN induction by the RLR pathway (Dudek et al., 2011; Varga et al., 2011).

4.3. The viral polymerase

PB1-F2 is not the only influenza viral protein that is found in the mitochondria. PB2, one of the subunits of the viral polymerase, is found not only in the nucleus, where RNA

replication takes place, but also in the mitochondria (Carr et al., 2006), where it appears to inhibit MAVS function too (Graef et al., 2010; Iwai et al., 2010). A role of the viral polymerase in inhibiting IFN was previously suggested by partial UV inactivation studies of influenza virus (Marcus et al., 2005), and by systems biology analysis of cellular proteins interacting with the viral proteins (Shapira et al., 2009). In addition, the cap snatching activity associated with the influenza virus polymerase, which generates primers for the synthesis of viral mRNA, is also likely to contribute to general inhibition of gene expression (Schmolke and García-Sastre, 2010). Also interestingly, virus strains with highly efficient polymerases can outrun the IFN response in vivo due to the high speed in replication (Grimm et al., 2007).

4.4. The M2 protein

The M2 protein of influenza virus is a small ion channel transmembrane protein that is needed for proper uncoating of influenza virus ribonucleoproteins during the viral entry process (Pinto et al., 1992). More recently, it was found that this protein also interferes with cellular autophagy (Gannage et al., 2009). As autophagy participates in TLR activation during viral infection (see above), it seems reasonable that M2 would then prevent this mechanisms of TLR induction. However, this remains to be investigated.

4.5. Use of cellular inhibitory factors

In addition to the direct role of several influenza virus proteins in inhibiting the IFN system, the virus is known to take advantage of specific cellular negative regulators of IFN to further decrease the antiviral effects of IFN. Influenza virus infection results in upregulation of SOCS factors involved in inhibition of IFN signaling (Jia et al., 2010; Pauli et al., 2008; Pothlichet et al., 2008). Influenza virus is also known to exploit the cellular PKR inhibitor P58(IPK) to indirectly inhibit PKR activation (Melville et al., 1999). This is mediated by influenza virus induced dissociation of P58(IPK) from Hsp40. Recently, Sharma et al have found that the viral NP is mediating this dissociation (Sharma et al., 2011), thus implicating then this viral protein in inhibiting PKR activation together with NS1.

5. Impact of the IFN system in influenza virus host tropism and pathogenesis

Despite the multiple inhibitory activities of different influenza virus proteins in the IFN response, IFN is still critical for prevention of severe influenza virus infection. Studies conducted in mice clearly indicate that in the absence of IFN, viral replication and the severity of the disease is increased, with both type I and type III IFN having protective roles (Mordstein et al., 2008). As most of the IFN antagonistic activities of the virus are due to interactions with cellular proteins, it is very likely that these interactions play a role in host tropism, as some strains may have been adapted for optimal inhibition of cellular factors involved in the IFN response in a specific host, as is the case with influenza B virus NS1 and ISG15. It has also become clear that the IFN antagonistic properties of the viral NS1 are required for virus replication and pathogenesis in vivo (García-Sastre et al., 1998). Also interestingly, the NS1 impacts not only innate immunity, but also subsequent adaptive immune responses (Haye et al., 2009), making NS1 mutant viruses attractive candidates as attenuated viral vaccines based on their attenuation and high immunogenicity (Richt and García-Sastre, 2009). Inhibition of NS1 function might also be an attractive strategy for the identification of small molecules with antiviral activity against influenza viruses. In this respect, we have recently identified a small molecule that restores cellular gene expression in the presence of NS1 and that inhibits viral replication (Mata et al., 2011). Future research will be required to find out whether some of these approaches can be developed into new vaccines and antivirals for the prevention and treatment of influenza virus infections.

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Figure 1. Induction of IFN by influenza virus

Influenza virus enters the cell through the endocytic pathway. Fusion of the envelope of the virus to that of the endosome results in the release of the segmented genome of the virus, which is transported to the nucleus where replication takes place. During this entry process, some virions may not escape the endosome and expose the viral RNA to TLR3/7/8 present in specialized cells, which recognize double stranded and single stranded RNA. At late times of infection, the newly synthesized viral RNA segments move to the cytoplasm, where they can encounter the ubiquitously expressed cytoplasmic sensor RIG-I before assembly of new virions takes place. The presence of a 5'-triphosphate and of a partial double strand structure makes the influenza virus RNAs optimal substrates for RIG-I activation. Viral RNAs in the cytoplasm can also enter the endosome through autophagic processes. TLR signaling by the adaptors TRIF or MyD88, and RIG-I signaling by the adaptor MAVS triggers signal transduction cascade that results in activation of IRF3/7 and NF-κB factors that translocates to the nucleus where they promote the synthesis of type I and type III IFN mRNAs.

Figure 2. Inhibition of IFN by influenza virus

Influenza virus inhibits the IFN system at different levels by using viral (black boxes) and cellular (grey boxes) proteins. The viral NS1 protein sequesters RNA from activating RIG-I, PKR and OAS. This protein also binds to and inhibits TRIM25 and PKR. In the nucleus, the NS1 prevents the proper processing of cellular mRNAs, inhibiting the synthesis of IFN and ISGs. Both PB1-F2 and PB2 proteins have been shown to inhibit MAVS function in the mitochondria. The NS1 protein of influenza B virus (BNS1) binds to and inhibits ISG15. Cellular factors SOCS and P58(IPK) are activated during viral infection to inhibit IFN signaling and PKR activation, respectively. Inhibitory actions are shown by a line that ends in a filled circle.