Influenza virus H5N1 non-structural protein 1 alters interferon-α/β signaling

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Immunology

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**ABSTRACT**

**Influenza virus H5N1 non-structural protein 1 alters interferon-α/β signaling**

Danlin Jia, Master of Science,
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Type I interferons (IFNs) function as the first line of defense against viral infections by modulating numerous biological processes to establish an antiviral state and influencing the activation of various immune cells. During influenza A infection, the NS1 encoded by the virus genome disrupts many cellular processes to block type I IFN responses. We show that expression of H5N1 NS1 in HeLa cells reduces IFN-inducible activation of STAT proteins and its subsequent binding to DNA complexes. Subsequent analysis suggests NS1 blocks IFN signaling by inhibiting expression of type I IFN receptor subunit, IFNAR1, as well as up-regulating SOCS1 expression. Finally, we demonstrate that pretreatment of primary human lung tissue with IFN alfacon-1 inhibits H5N1 viral replication by up-regulating a number of interferon-stimulated genes. The data suggest that NS1 can directly interfere with Type I IFN signaling, and that pretreatment with IFN can inhibit H5N1 infection in primary human lung tissue.
ACKNOWLEDGEMENT

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<th>Description</th>
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<tr>
<td>2-5A</td>
<td>2'-5’ oligoadenylate</td>
</tr>
<tr>
<td>2’5’OAS</td>
<td>2’-5’oligoadenylate synthetase</td>
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<tr>
<td>4E-BP1</td>
<td>eIF4E-binding protein</td>
</tr>
<tr>
<td>AP-1</td>
<td>activation protein-1</td>
</tr>
<tr>
<td>APRE</td>
<td>acute phase response element</td>
</tr>
<tr>
<td>BRG</td>
<td>Brahma-related gene</td>
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<td>BAF</td>
<td>BRG-BRM-associated factor</td>
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<td>CARD</td>
<td>caspase-recruitment domains</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CID</td>
<td>central interactive domain</td>
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<tr>
<td>CIS</td>
<td>cytokine-inducible SH2-containing</td>
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<tr>
<td>CPSF</td>
<td>cleavage and polyadenylation specificity factor</td>
</tr>
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<td>CrkL</td>
<td>V-crk sarcoma virus CT10 oncogene homolog (avian)-like</td>
</tr>
<tr>
<td>c-Src</td>
<td>cellular sarcoma</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DsRNA</td>
<td>double stranded ribonucleic acid</td>
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<tr>
<td>eIF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
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<tr>
<td>ERK</td>
<td>extracellular signal regulated kinases</td>
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<tr>
<td>FADD</td>
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<tr>
<td>FBN</td>
<td>fibronectin</td>
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<tr>
<td>GAS</td>
<td>γ-activating sequence</td>
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<tr>
<td>GCN5</td>
<td>general-control-amino-acid synthesis 5</td>
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<tr>
<td>GEF</td>
<td>guanine exchange factor</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
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<td>histone acetyl transferases</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HTLV</td>
<td>human T-cell leukemia virus</td>
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<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of NFκB</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>ISG</td>
<td>interferon-stimulated gene</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>LGP2</td>
<td>likely ortholog of mouse D11lgp2</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
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<tr>
<td>LZ</td>
<td>leucine zipper</td>
</tr>
<tr>
<td>M</td>
<td>matrix</td>
</tr>
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<td>MAL</td>
<td>MyD88 adaptor-like</td>
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<td>mitogen-activated protein kinase</td>
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<td>MAKK</td>
<td>MAK kinase</td>
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<tr>
<td>MAKKK</td>
<td>MAKK kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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<tr>
<td>MDA5</td>
<td>melanoma differentiation antigen 5</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>Mx</td>
<td>Orthomyxovirus resistance</td>
</tr>
<tr>
<td>NA</td>
<td>neuraminidase</td>
</tr>
<tr>
<td>ND</td>
<td>nuclear domain</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>nonstructural</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>PAB</td>
<td>polyadenylate binding protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIAS</td>
<td>protein inhibitor of activated STAT</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PML NB</td>
<td>promyelocytic leukemia protein nuclear body</td>
</tr>
<tr>
<td>PRD</td>
<td>positive regulatory domain</td>
</tr>
<tr>
<td>PRE</td>
<td>prolactin response element</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RD</td>
<td>repressor domain</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic-inducible gene I</td>
</tr>
<tr>
<td>RLH</td>
<td>RIG-like helicase</td>
</tr>
<tr>
<td>S6K</td>
<td>S6 kinase</td>
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<tr>
<td>SARM</td>
<td>sterile α- and armadillo-motif-containing protein</td>
</tr>
<tr>
<td>SIE</td>
<td>sis-inducible element</td>
</tr>
<tr>
<td>SH</td>
<td>src-homology</td>
</tr>
<tr>
<td>SOC</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-related modifier</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK1-binding protein</td>
</tr>
<tr>
<td>TAD</td>
<td>transcriptional activation domain</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF-family-member-associated NFκB activator</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>TCP</td>
<td>T cell-PTP</td>
</tr>
<tr>
<td>THOV</td>
<td>Thogotovirus</td>
</tr>
<tr>
<td>TIR</td>
<td>toll/interleukin 1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR-associated protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TOP</td>
<td>terminal oligopyrimidine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor (TNF) receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor protein-inducing IFN-β</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>Ubc13</td>
<td>ubiquitin conjugating enzyme 13</td>
</tr>
<tr>
<td>Uev1A</td>
<td>ubiquitin-conjugating E2 enzyme variant 1A</td>
</tr>
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</table>
CHAPTER I
INTRODUCTION

IFN was discovered by Isaac and Lindenmann in 1957 as a secreted substance that confers antiviral activity against influenza infection (Isaacs and Lindenmann, 1957). The discovery of IFNs paved the way for understanding other class II cytokines and their receptors. IFNs are pleiotropic cytokines that are produced in response to viral challenge, and function to protect the host against infection via the transcriptional and translational induction of a series of proteins that interfere with different stages in the replicative cycle of viruses (Samuel, 1991). In addition, IFNs activate a number of immune cells, thereby invoking the clearance of virus (Le Bon and Tough, 2002).

I.1 Types of IFN

There are three types of IFN, each characterized by their distinct cognate receptors. The Type I IFNs are comprised of different subtypes including: multiple IFN-α subtypes (14 human, 11 mouse), IFN-β, IFN-ε, IFN-ω, IFN-δ and IFN-τ (Hardy et al., 2004). Type I IFNs can be produced by most cell types and bind as monomers to the two subunits of the type I IFN alpha receptor, IFNAR1 and IFNAR2 (de Weerd et al., 2007). IFN-γ is the sole Type II IFN, is functionally active as a dimer, and binds with high affinity to its cognate receptor that is comprised of the two subunits IFNGR1 and IFNGR2 (Soh et al., 1994; Soh et al., 1993). Unlike type I IFNs, IFN-γ is only produced by few cell types, including natural killer (NK) cells, CD4+ T helper 1 (Th1) cells, and dendritic cells (DC). The third type of IFN has 3 members, IFNλ1, IFNλ2 and IFNλ3, also known as interleukin-29 (IL-29), IL-28A, and IL-28B, respectively (Kotenko et al.,
Type III IFNs bind with high affinity to the IFN lambda receptor subunits, IFNLR1 and IFNLR2. Type I and type II IFNs have distinct and overlapping signaling pathways as well as biological activities. Current evidence suggests that type III IFNs share many of the signaling and responses of type I IFNs (Dumoutier et al., 2003). Different type I IFNs exhibits differential affinity for its receptor, but currently the IFN that has the highest affinity for type I IFN receptors belong to the recombinant IFN named IFN-alfa-con-1 (Ozes et al., 1992). This IFN was generated by comparing the most frequent occurring amino acid among endogenous type I IFNs (Pfeffer, 1997).

### 1.2 Induction of type I IFNs

Type I IFNs are rapidly induced when viral or bacterial derived factors, also known as pathogen-associated molecular patterns (PAMPs) interact with cellular pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997). PRRs include members of the toll-like receptor (TLR) family, cytosolic sensors like retinoic acid-inducible gene I (RIG-I)-like helicase (RLH), nucleotide-oligomerization domain (NOD)-like receptors (NLRs) and the DNA-dependent activator of IRFs, DAI. PRR activation leads to downstream signalling cascades and the production of type I IFNs and/or other pro-inflammatory cytokines (Kawai and Akira, 2006). During the late 1980s, Charles Janeway hypothesized that PRRs would be capable of detecting a broad range of infectious agents, and subsequently induce appropriate immune responses to combat infection. This concept led to the subsequent identification and characterization of PAMPs, shared across different microbial families (Medzhitov and Janeway, 1997). These conserved moieties are not only “foreign” to the host, enabling discrimination from
self, but also represent molecules that are intolerant to extensive mutations because of their critical roles in the clearance of microbes.

I.2.1 Toll-like Receptors

Toll, a protein involved in Drosopila embryogenesis, was found to play a critical role in the immune response to fungus infection (Medzhitov et al., 1997). This led to the subsequent discovery and characterization of its mammalian homologue and related family members. There are currently 10 human TLRs and 13 murine TLRs (Takeda et al., 2003). Each TLR acts alone or in combination with other TLRs to detect unique PAMPs. TLR4 was first shown to be involved in the recognition of lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls (Poltorak et al., 1998). TLR1, in combination with TLR2, recognizes triacyl lipopeptides, whereas when complexed with TLR6, TLR2 can also bind diacyl lipopeptide (Hajjar et al., 2001; Shimizu et al., 2005). TLR3 is the receptor for double stranded RNA (dsRNA) (Alexopoulou et al., 2001). TLR5 is activated in the presence of bacterial flagellin (Hayashi et al., 2001). TLRs7/8 recognize ssRNA, whereas TLR9 binds to unmethylated dsDNA (Heil et al., 2004; Hemmi et al., 2002; Takeshita et al., 2001). TLRs are also differentially located in cells, with TLR1, 2, 4, 5 and 6 found on the plasma membrane and TLR3, 7, 8 and 9 located inside late endosomes or lysosomes (Kawai and Akira, 2005). TLRs are type I transmembrane proteins, each composed of extracellular leucine-rich repeats (LRRs) followed by one or two cysteine-rich regions which are involved in ligand binding. TLRs have short transmembrane domains connected to a cytoplasmic toll/interleukin 1 receptor
homology (TIR) domain, which functions in the recruitment of downstream adaptor and signaling components in response to ligand recognition (Akira, 2004).

### I.2.1.1 TLR signaling activates type I IFN production

TLR-ligand interactions lead to changes in receptor conformation, allowing the cytoplasmic TIR domain to interact with several downstream TIR-containing adaptor proteins, including MyD88, MyD88 adaptor-like (MAL or TIR-associated protein [TIRAP]), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF or TIR-domain-containing molecule 1 [TICAM1]), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007). Differential usage of these adaptors can result in different downstream responses, which are distinguished by two pathways: the MyD88-dependent pathway and the MyD88-independent pathway. All but TLR3 can activate the MyD88-dependent pathway, whereas only TLR3 and TLR4 can signal through the MyD88-independent cascade (Kawai and Akira, 2005; Oshiumi et al., 2003; Yamamoto et al., 2002). Notably, only TLR3, 4, 7, 8 and 9 activation leads to the production of type I IFNs (Figure I.1). Current evidence suggests stimulation of TLR1, 2, 5 and 6 leads to signaling through the classical MyD88-dependent nuclear factor-kappa B (NFkB) pathway, resulting in the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) but not IFNs (Bas et al., 2008; Fisette et al., 2003; Wang et al., 2001b; Zeng et al., 2006).
I.2.1.2 TLR4

TLR4 is expressed on the cell surface, and its extracellular portion associates with MD-2, a secreted polymeric protein required for the oligomerization of TLR4 in response to LPS. MD-2 is also involved in the glycosylation of TLR4, a necessary process for its translocation to the plasma membrane (Shimazu et al., 1999). In addition, TLR4-MD2 can associate with CD14, a co-receptor for LPS (Jiang et al., 2005). Upon ligand activation, the TLR4-MD2-CD14 complex recruits both adaptor MAL and TRAM, initiating both MyD88-dependent and MyD88-independent signaling, respectively (Seya et al., 2005). On one hand, in the absence of MyD88, TRAM can interact with the adaptor TRIF, which in turn leads to association with the tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) (Hoebe et al., 2003; Yamamoto et al., 2003b). TRAF6 subsequently activates TRAF-family-member-associated NFκB activator (TANK)-binding kinase 1 (TBK1) and the non-canonical inhibitor of NFκB (IκB) kinase (IKKi) (Hemmi et al., 2004; Sato et al., 2003). TBK1 will phosphorylate interferon regulatory factor 3 (IRF3), allowing it to dimerize and translocate to the nucleus, where it can induce the transcription of IFN-β (Sakaguchi et al., 2003). TLR4 can also interact with MyD88 via the TIR domain through the adaptor MAL, which leads to the downstream activation of the interleukin-1 receptor (IL-1R) associated kinase -4 and -1 (IRAK-4 and IRAK-1), TRAF6 and TGF-β-activated kinase 1 (TAK1) (Dong et al., 2006; Fitzgerald et al., 2001; Loiarro et al., 2007; Muzio et al., 1997; Suzuki et al., 2002). Though understanding the precise relationship between these kinases requires ongoing investigation, current evidence suggests binding of MyD88 brings IRAK4, IRAK1 and TRAF6 together at the receptor complex. IRAK4 subsequently phosphorylates IRAK1, leading to its activation
and autophosphorylation (Cheng et al., 2007; Kollewe et al., 2004; Li et al., 2002). Hyperphosphorylated IRAK1 dissociates from MyD88 to form a cytoplasmic complex with TRAF6 via interactions between their death domains (DD)(Ahmad et al., 2007; Qian et al., 2001). TRAF6 in turn interacts with a multiprotein complex including TAK1-TAK1-binding protein 1 (TAB1)-TAB2 and ubiquitin-conjugation enzyme 13 (Ubc13) and ubiquitin-conjugating E2 enzyme variant 1A(Uev1A)(Deng et al., 2000; Takaesu et al., 2000). TAK1 activation leads to phosphorylation of the IKK complex (including IKKα, IKKβ and scaffolding IKKγ), which subsequently leads to the phosphorylation of IκB and its degradation(Lee et al., 2000; Takaesu et al., 2003; Wang et al., 2001a). This in turn facilitates the release and translocation of NFκB into the nucleus. TAK1 can also phosphorylate mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinase (ERK) 1 and 2, c-Jun N-terminal kinases (JNKs) and p38, leading to the activation of activation protein-1 (AP-1), which can function cooperatively with either IRF3 or NFκB to induce the expression of type I IFNs or proinflammatory cytokines, respectively (Thiefes et al., 2005; Wang et al., 2001a; Yang et al., 2004).
Figure 1.1 Induction of type I IFNs. Viral derived-factors or PAMP (red arrow) activate PRRs like TLRs (TLR3,4,7,8,9) and cytoplasmic sensors (RIG-I, MDA5, DAI) to induce transcriptional activation of type I IFNs mediated by IRF3 and IRF7.
I.2.1.3 TLR3

TLR3 is predominantly located in endosomes and phagosomes, the exceptions being TLR3 expression on the cell surface of epithelial and natural killer (NK) cells (Matsumoto et al., 2003; Schmidt et al., 2004; Xie et al., 2007). TLR3 recognizes dsRNA, a common replicative intermediate during virus infection. Evidence suggests acidification of endosomes is the first step toward initiating TLR3 activation (de Bouteiller et al., 2005). Downstream signaling from TLR3 is mediated in a MyD88-independent fashion, with recruitment of TRIF first to its TIR domain, followed by aggregation with TRAF3 (Oganesyan et al., 2006; Yamamoto et al., 2003a). TRAF3 in turn activates kinases TBK1 and IKKi, leading to the subsequent phosphorylation of IRF3, which dimerizes and translocates to the nucleus to activate IFN-β gene expression (Hacker et al., 2006; Yoneyama et al., 1998). In addition, TLR3-dsRNA interactions induce phosphorylation of TLR3 cytoplasmic tyrosine residues, which then function as docking sites for phosphatidylinositol 3-kinase (PI3K) (Johnsen et al., 2006; Sarkar et al., 2004). Activation of PI3K in response to dsRNA is required for the complete phosphorylation of IRF3 (Dong et al., 2008). Furthermore, tyrosine kinase cellular sarcoma (c-Src) kinase can also be activated and associate with TLR3 in response to dsRNA, though its role in downstream signaling is yet to be determined (Johnsen et al., 2006).

I.2.1.4 TLR7/8/9

Human plasmacytoid dendritic cells (pDC) lack TLR3 and TLR4, yet express high levels of TLR7 and TLR9, whose activation leads to robust type I IFN production in response to virus-derived single stranded RNA (ssRNA) or unmethylated CpG DNA
motifs, respectively (Hornung et al., 2002). TLR7, 8 and 9 invoke the MyD88-dependent signaling pathway (as described above) to induce both type I IFNs and proinflammatory cytokines. In contrast to TLR3, the induction of type I IFNs from these TLRs does not require the presence of IRF3 (Honda et al., 2005). Following ligand activation, MyD88, IRAK4, IRAK1 and TRAF6 recruitment to the receptor results in activation of downstream TAK1. This leads to the activation and subsequent nuclear translocation of NFκB and AP-1. In addition, TRAF3 can also aggregate with MyD88, IRAK1, IKKα and precursor of osteopontin (OPN) to activate IRF7 (Honda et al., 2004; Kawai et al., 2004; Oganesyan et al., 2006). Phosphorylated IRF7 can subsequently dimerize and translocate to the nucleus, where it interacts with the promoters in the IFN-α and IFN-β genes to activate their transcription (Kawai et al., 2004; Yeow et al., 2000). For both TLR8 and TLR7, ligand recognition overlaps, thereby confounding the identification of distinguishing signaling pathways. TLR8 may function as a negative regulator for TLR7 and TLR9 (Wang et al., 2006b).

I.2.2 RLH

The induction of type I IFNs in the absence of TLRs led to the identification of TLR-independent viral sensors. The RNA helicase RIG-I was the first cytoplasmic receptor identified capable of sensing dsRNA and activating type I IFN production (Yoneyama et al., 2004). RIG-I is comprised of a C-terminal DExD/H box RNA helicase domain that interacts with dsRNA in an ATP-dependent manner, two N-terminal caspase-recruitment domains (CARDs) which can interact with other CARD-containing proteins, and a repressor domain (RD), that has been shown to suppress signaling in the resting
state (Saito et al., 2007). Two other members of the RIG-I-like helicases (RLH) have been described: melanoma differentiation antigen 5 (MDA5) and likely ortholog of mouse D11lgp2 (LGP2). Like RIG-I, MDA5 contains two CARD-like domains and a helicase domain. In contrast, LGP2 only possesses the helicase domain and does not contain a CARD-like domain. LGP2 may function as a negative regulator of RIG-I and MDA5 (Yoneyama et al., 2005). Notably, RIG-I and MDA5 exhibit some specificity in the viral PAMPs they recognize. Gene targeting studies have shown that RIG-I is critical for the detection of paramyxoviruses, vesicular stomatitis virus (VSV) and influenza viruses, whereas MDA5 is vital for the recognition of picornaviruses (Childs et al., 2007; Kato et al., 2006; Loo et al., 2008).

I.2.2.1 Type I IFN production mediated by RLH signaling

Upon binding to target RNA, RIG-I first undergoes ubiquitination of its CARD domain. This ubiquitin E3 ligase tripartite motif 25 (TRIM25) catalyzed reaction is critical for efficient downstream signalling (Gack et al., 2007). In contrast, ubiquitination by another protein, RNF125, targets RIG-I for degradation, suggesting RIG-I-mediated signaling is tightly regulated through differential ubiquitination (Arimoto et al., 2007). Subsequent to ligand recognition, RIG-I undergoes a conformational change and self-association, which in turn leads to binding with its downstream adaptor IFN-β promoter stimulator 1 (IPS-1, also known as MAVS, VISA or CARDIF) via CARD-CARD interactions. IPS-1 is comprised of an N-terminal CARD domain, a proline rich region and a hydrophobic transmembranous (TM) region at the C-terminus (Kawai et al., 2005). The TM region anchors IPS-1 to the outer membrane of the mitochondrion, required for
subsequent signaling (Seth et al., 2005). Activated IPS-1 can associate with TRAF3 to activate downstream kinases TBK1 and IKKi, which in turn can phosphorylate IRF3 and IRF7 to induce type I IFN gene expression (Kawai et al., 2005; Xu et al., 2005). IPS-1 can also interact with Fas-associated death domain-containing protein (FADD), a mediator in death receptor signalling (Kawai et al., 2005). FADD associates with caspase-8 and caspase-10, leading to their cleavage and the activation of downstream NFκB, resulting in the production of pro-inflammatory cytokines (Kreuz et al., 2004). However, the precise mechanism leading to NFκB activation from caspase cleavage remains to be determined. In addition to the RLH family, there are other cytoplasmic sensors that can respond to PAMPs. DNA-dependent activator of IRFs (DAI) was recently identified to induce an IFN response when stimulated with dsDNA, and its presence seems to be important for detecting DNA viruses (Takaoka et al., 2007).

I.2.3 IRF in type I IFN induction

The IRF family of proteins is comprised of nine members (Taniguchi et al., 2001). IRF3 and IRF7 play key roles in mediating type I IFN gene expression. IRFs possess a conserved N-terminal DNA binding domain (DBD) with five tryptophan repeats (Harada et al., 1994; Taniguchi et al., 2001; Veals et al., 1992). Crystal structure studies have identified 5’-AANNGAAA-3’ as the consensus base sequence recognized by IRFs (Fujii et al., 1999; Tanaka et al., 1993). Binding to this sequence leads to changes in DNA structure that may allow cooperative binding of other transcription factors like AP-1 and NFκB to nearby target sequences. Under normal conditions, both IRF3 and IRF7 reside in the cytoplasm in inactive forms. Upon PRR activation, IRF3 and IRF7 undergo serine
phosphorylation mediated by activated TBK1 and IKKi (as described above), and form either homodimeric or heterodimeric complexes (Fitzgerald et al., 2003; Hemmi et al., 2004; Sharma et al., 2003). These dimers translocate to the nucleus and complex with other co-activators to target specific gene elements in the promoters of type I IFNs and other cytokines, thereby activating transcription (Marie et al., 2000). In contrast to IRF3, which plays a major role in regulating IFN-β expression, IRF7 can activate gene expression of the IFN-αs and IFN-β. IRF7 is generally less abundant in cells than IRF3, with the exception of cells of lymphoid origin, particularly plasmacytoid dendritic cells (pDC) (Izaguirre et al., 2003). The expression of IRF7 can be up-regulated in response to IFN-β stimulation, which in turn can act to induce gene expression for the IFN-αs. Maintenance of this positive feedback loop is thought to require the ongoing presence of viral factors, since IRF7 has a short half-life due to its susceptibility to ubiquitin-mediated degradation (Negishi et al., 2005). Other IRF members such as IRF1 and IRF5 have also been implicated in type I IFN production, though gene targeting studies suggest that both are dispensable for normal IFN expression (Reis et al., 1994; Schoenemeyer et al., 2005).

I.3 Transcriptional regulation of type I IFNs

Transcription of the IFN-β gene requires the assembly of the transcriptional complex, also known as the enhanceosome, at the enhancer region upstream of the IFN-β gene transcription start site. The enhancer region of the IFN-β gene contains four positive regulatory domains (PRDs I, II, III and IV), whereas genes for the IFN-αs contain PRD-I- and PRD-III-like elements (PRD-LE) (Kim and Maniatis, 1997; Ryals et al., 1985).
Activated IRFs recognize PRD-I and PRD-III, whereas PRD-II is targeted by AP-1 and PRD-IV by NFκB. These activated transcriptional factors associate with the high-mobility group protein I (Y) (HMG I [Y]) to form the enhanceosome (Kim and Maniatis, 1997). The enhanceosome subsequently recruits histone acetyl transferases (HATs), including general-control-control-amino-acid synthesis 5 (GCN5) and CREB-binding protein (CBP/p300) to catalyze histone (H3 and H4) acetylation. This modification results in engagement of Brahma-related gene (BRG)-Brahma (BRM)-associated factor (BAF) complex, which leads to spatial alternation in the nucleosome and facilitates the binding of the RNA polymerase complex to the start site of transcription (Agalioti et al., 2000).

1.4 Type I IFN receptors

Following PRR activation and the subsequent induction of expression for Type I IFNs, these secreted proteins function in both autocrine and paracrine ways to influence multiple cellular functions to establish an antiviral state. For Type I IFNs to exert their influence in cells, the absolute requirement is that these cells express the two transmembrane receptor subunits of the IFN receptor, IFNAR: IFNAR1 and IFNAR2c. These receptors were identified through a series of genetic cloning and IFN sensitivity reconstitution assays using somatic cell hybrids (Langer and Pestka, 1988). In contrast to IFNAR1, human IFNAR2 has three isoforms as a result of alternative splicing and differential usage of exons and polyadenylation. IFNAR2c represents the isoform comprised of an extracellular domain, a transmembrane region and a cytoplasmic domain (Lutfalla et al., 1995). In contrast, IFNAR2a is a soluble receptor. IFNAR2b is similar to
IFNAR2c, but lacks a cytoplasmic domain (Novick et al., 1995). Interestingly, mice do not have the IFNAR2b isoform, instead there are two soluble IFNAR2a murine variants, arising from differential splicing (Owczarek et al., 1997). Binding to and engagement of both IFNAR1 and IFNAR2c are required for productive type I IFN signalling. The type I IFNs bind with higher affinity to IFNAR2 compared with IFNAR1, and IFNAR2c is considered the primary binding receptor subunit (Jaks et al., 2007). The extracellular portion of IFNAR1 is comprised of four domains named SD 1-4, with each domain containing a fibronectin (FBN) III-like motif. SDs 1-3 appear to contribute to ligand binding, whereas SD4 appears critical for the formation of the receptor complex (Ghislain et al., 1994; Lamken et al., 2005). On the other hand, all isoforms of IFNAR2 contain two FBN-like domains configuring as an immunoglobulin-like folding structure (Chill et al., 2003; Kumaran et al., 2007; Runkel et al., 2000). The binding of type I IFNs to these cognate receptors leads to activation of receptor-associated Janus kinases (JAKs), which in turn phosphorylate tyrosine residues in the intracellular domains of each receptor subunit, leading to the recruitment of signaling effectors, their phosphorylation-activation and subsequent activation of signaling cascades. In contrast to the non-signaling receptor subunit IFNAR2b, soluble IFNAR2a is capable of acting as both activator and inhibitor in the context of IFN signaling, yet more studies are required to elucidate its precise function (Fernandez-Botran, 1991; Han et al., 2001; Hardy et al., 2001).

I.5 Type I IFN signaling

Binding of type I IFNs to IFNAR leads to receptor aggregation and activation of receptor-associated kinases: JAKs. The JAK family is comprised of JAK1, 2, 3 and
TYK2, each containing a protein kinase domain at their carboxyl-terminus and five other domains that make up the N-terminus (Darnell et al., 1994; Stark et al., 1998). TYK2 and JAK1 interact with the cytoplasmic domains of IFNAR1 and IFNAR2, respectively. Activation of these kinases leads to phosphorylation of cytoplasmic tyrosine residues in the IFNARs, thereby generating docking sites for src-homology 2 (SH2)-containing signaling molecules (Platanias and Colamonici, 1992). The recruitment and phosphorylation of downstream molecules initiates a series of signaling pathways, leading to both transcriptional and translational activation (Figure I.2).

1.5.1 JAK-STAT pathway

Proteins that play a prominent role in mediating the transcriptional activation of type I IFNs are the signal transducers and activators of transcription (STAT) proteins (Figure I.2). There are seven members in this family: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Copeland et al., 1995; Fu et al., 1992; Schindler et al., 1992). STAT proteins, by their name, can relay signals from a wide spectrum of cytokine-receptor complexes, including IFN-, interleukin- and growth factor-receptor complexes (Bromberg, 2001; Schindler, 2002). Each STAT has a dimerization domain at the N-terminus, a coil-coil domain, a DNA binding domain, a linker domain, an SH2 domain and a carboxyl transcriptional activation domain (TAD) (Levy and Darnell, 2002). STATs were first thought to exist as monomers in the cytoplasm in the absence of receptor stimulation, but recent evidence indicates that many STATs form dimers and shuttle between the cytoplasm and nucleus even without cytokine-receptor activation.
Figure I.2 Type I IFN signaling. Binding of type I IFNs to their cognate receptors leads to activation of receptor-associated kinases Jak1 and Tyk2, and initiates both transcriptional and translational activation to establish an antiviral response.
(Koster and Hauser, 1999; Mao et al., 2005). In the presence of type I IFNs, STAT dimers are recruited to phosphorylated IFNARs through their SH2 domains and become phosphorylated by JAKs (Shuai et al., 1993; Silvennoinen et al., 1993). This tyrosine-phosphorylation leads to STAT homo- or hetero-dimerization, mediated by the binding of the SH2 domain of one STAT to the phospho-tyrosine of another (Becker et al., 1998; Chen et al., 1998; Mao et al., 2005). Activated STAT dimers subsequently enter into the nucleus via importin-mediated translocation, and target specific DNA elements to activate the expression of IFN-stimulated genes (ISGs) (Friedman et al., 1984; Larner et al., 1984; McBride et al., 2002). A number of STAT complexes are formed in response to type I IFN stimulation, with IFN stimulated gene factor 3 (ISGF3) being one of the best characterized complexes (Fu et al., 1990; Levy et al., 1989). ISGF3 is comprised of STAT1, STAT2 and IRF9/p48. Nuclear translocation enables ISGF3 to target gene promoters containing an IFN stimulated response element (ISRE), to activate the expression of ISGs including ISG15, 6-16 and ISG54 (Darnell et al., 1994). Though complexes like STAT2:2:IRF9 and STAT2:6:IRF9 can also target ISREs in the promoters of genes, they exhibit a much lower binding affinity when compared to ISGF3. In addition, other activated complexes like STAT1:1, STAT1:3, STAT3:3 and STAT1:2 target another group of ISGs whose promoters contain the consensus γ-activating sequence (GAS) element (Brierley and Fish, 2005a; Ghislain and Fish, 1996; Vinkemeier et al., 1996). Variants of GAS elements include acute phase response element (APRE), sis-inducible element (SIE) and prolactin response element (PRE) (Decker et al., 1997). Activation of ISG expression functions to inhibit virus infection mediated by different antiviral proteins. STAT signaling is negatively regulated by members of the cytokine-
inducible SH2-containing protein (CIS)/suppressor of cytokine signaling (SOC) family, protein inhibitors of activated STAT (PIAS) and other protein tyrosine phosphatases (PTPs) (Liu et al., 2004; Song and Shuai, 1998). SOC family members contain a variable N-terminus, an SH2 domain, and a SOCS box domain at the carboxy terminus (Hilton et al., 1998; Krebs and Hilton, 2000). Though there are eight SOC family members, only SOC1, SOC3 and CIS are well characterized for their role in regulating type I IFN signaling. SOC1 inhibits IFN signaling through direct physical interaction with JAK, whereas SOC3 and CIS interact with the phosphorylated receptor to hinder the recruitment and phosphorylation of downstream mediators like STAT proteins (Matsumoto et al., 1999; Yasukawa et al., 1999). In contrast to SOCS whose expression are rapidly induced post cytokine stimulation, PIAS proteins are constitutively expressed and interact directly with activated STATs to block their DNA-binding activity (Liu et al., 1998; Starr et al., 1997). Other phosphatases like SH2 domain-containing PTP-2 (SHP-2) and T cell-protein tyrosine phosphatase (Tc-PTP) 45 (TCP45) have also been shown to inactivate STATs through dephosphorylation (Simoncic et al., 2002; Wang et al., 2006a).

I.5.2 PI3K and Akt pathway

Type I IFNs can also trigger the activation of the PI3K pathway to modulate cellular translation and survival. Upstream effectors in this signaling cascade are the adaptor protein insulin receptor substrates (IRSs). IRSs were originally identified as critical mediators of insulin signalling (Myers et al., 1993; White, 1998). They contain residues that can undergo phosphorylation to become docking sites for downstream SH2-containing signaling moieties (Platanias et al., 1996). IFN-α/β-receptor interactions result
in IRS-1 and IRS-2 phosphorylation by JAK1, leading to the interaction between IRS-1 and the regulatory subunit of PI3K, p85, through its SH2 domain (Burfoot et al., 1997). Phosphorylation of p85 activates the catalytic subunit of PI3K, p110, which can in turn activate one of its downstream effectors, protein kinase B (PKB)/Akt, through the generation of phosphatidylinositol 3, 4, 5-triphosphate (PIP_3) (Manning and Cantley, 2007; Uddin et al., 1997). Activation of PKB influences numerous cellular processes including both proliferation and survival (Manning and Cantley, 2007; Martelli et al., 2007).

1.5.3 PI3K and mammalian target of rapamycin (mTOR) pathway

IFN-inducible activation of PI3K can regulate protein translation by modulating the activity of the mTOR pathway (Manning and Cantley, 2003). Indeed, the Akt pathway has a role in mRNA translation (Kaur et al., 2008). Activated mTOR signals through two major downstream effectors, namely, the S6 kinase (S6K) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) to modulate protein translation (Hara et al., 1997; von Manteuffel et al., 1997). Through a physical association, 4E-BP1 blocks the interaction of eIF4E with other translation initiation factors, thereby preventing cap-dependent translation (Scheper et al., 1992). Phosphorylation of 4E-BP1 by mTOR relieves its association with eIF4E, thereby allowing its association with eIF4G and other co-factors to initiate translation (Hara et al., 1997). mTOR also influences translation through the activation of S6K. Activated S6K phosphorylates the ribosomal protein S6 to increase the translation of 5’-terminal
oligopyrimidine (TOP) mRNAs, which include ribosomal proteins and elongation factors (Jefferies et al., 1997; Tang et al., 2001).

I.5.4 V-crk sarcoma virus CT10 oncogene homolog (avian)-like (CrkL) pathway

CrkL belongs to the Crk family of proteins which include CrkI and CrkII. Crk proteins contain both SH2 and SH3 domains and function as adaptors in cytokine signalling (Mayer et al., 1988). IFN-α/β binding to IFNAR results in the activation of TYK2, leading to the phosphorylation of Casitas B-lineage lymphoma (CBL), an adaptor protein that associates constitutively with TYK2 (Uddin et al., 1996). Phosphorylated CBL acts to recruit CrkL via its SH2-binding domain. CrkL subsequently recruits a range of downstream effectors including C3G, a guanine exchange factor (GEF) for Rap-1 (Feller et al., 1995; Reedquist et al., 1996; Sattler et al., 1996; Tanaka et al., 1994). Rap-1, identified first as a tumor suppressor gene, inhibits the activity of the small GTPase Ras, to hinder cellular proliferation (Cook et al., 1993). CrkL can form a complex with phosphorylated STAT5 through its SH2 domain to activate the expression of GAS-containing genes (Fish et al., 1999).

I.5.5 Mitogen-activated protein kinase (MAPK) p38 pathway

The three major MAPK families are extracellular signal regulated kinases (ERKs), JNKs, and the p38 MAP kinases (Schaeffer and Weber, 1999). These serine-threonine kinases respond to various stimuli and coordinate numerous signaling cascades to generate appropriate cellular responses (Kyriakis and Avruch, 1996). The p38 MAP kinase family is comprised of four p38 isoforms (α, β, γ and δ) that can be activated in
response to stress (radiation, heat shock and hyperosmolarity), and cytokines such as interleukin-1 (IL-1), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) (Jiang et al., 1996; Jiang et al., 1997; Lechner et al., 1996; Lee et al., 1994; Raingeaud et al., 1995). Evidence from various studies suggests that upon IFN stimulation, Vav, a GEF, is phosphorylated by TYK2, and will activate the small G-protein Ras-related C3 botulinum toxin substrate 1 (Rac1) (Crespo et al., 1997; Platanias and Sweet, 1994). Rac1 activation initiates a series of downstream phosphorylation cascades involving MAPK kinase kinase (MAPKKK) and MAPK kinase (MAPKK), including MKK3/4/6 to activate p38 MAP kinases (Salojín et al., 1999). p38 MAPKs target other molecules such as MapKapK-2 and MapKapK-3, two serine kinases which have important roles in mediating the antiviral effect of type I IFNs (Mayer et al., 2001; Uddin et al., 1999). Although there have been reports that p38 MAPK may phosphorylate serine residues on STAT1, protein kinase C-δ (PKC-δ) is primarily responsible for the IFN-α/β inducible serine phosphorylation of STATs (Kovarik et al., 1999; Uddin et al., 2002). Phosphorylation of serine 727 on STAT1 and STAT3 is required to achieve their full transcriptional activation (Wen et al., 1995; Zhu et al., 1997).

I.6 Antiviral effectors

Type I IFNs are pleiotropic cytokines that influence numerous cellular processes. One of the major downstream responses is to generate effectors that inhibit the replication of pathogens. IFN-inducible transcriptional activation of ISGs and IFN-inducible regulation of translational events lead to a defined set of proteins being expressed that can exert inhibitory effects at different stages of viral replication to achieve an antiviral effect.
I.6.1 dsRNA-dependent protein kinase R

dsRNA-dependent protein kinase R (PKR) is one of the well characterized ISG products that participates in both IFN-inducible antiviral and antiproliferative responses. PKR is a serine-threonine kinase comprised of a kinase domain and two dsRNA binding domains (dsRBD) (Patel and Sen, 1992). PKR is activated by dsRNA, a common replicative intermediate during virus infection. Binding of dsRNA to PKR leads to PKR dimerization and subsequent autophosphorylation (Galabru and Hovanessian, 1987). This activated PKR acts on downstream effectors to modulate both translation and transcription. One of the well known targets of PKR is eIF2α, a factor that plays a critical role during the initiation of translation (Rhoads, 1993; Williams, 1999). eIF2 is made up of three subunits: α, β and γ, that together function to promote the guanine trisphosphate (GTP)-dependent delivery of Met-transfer RNA (tRNA) to the 40S ribosome during protein synthesis (Hershey, 1991). Next, GTP is hydrolyzed and allows eIF2 to dissociate from the initiation complex (Majumdar and Maitra, 2005). Subsequently, eIF2B, a GEF, will recycle the inactive eIF2-GDP back to its active form. However, activation of PKR leads to phosphorylation of eIF2α, which leads to an increase in its affinity for eIF2B. The sequestration of eIF2B results in the inhibition of Met-tRNA delivery and thereby prevents the initiation of translation of both viral and cellular mRNAs (Hershey, 1991).

In addition, PKR can modulate the activity of transcription factors in response to dsRNA. PKR plays a role in the phosphorylation of serine residues in STAT1 and STAT3 (Ramana et al., 2000). Abrogation of this PKR-mediated serine phosphorylation leads to loss of function of these STATs (Deb et al., 2001; Lee et al., 2005). PKR has also been
reported to participate in the activation of NFκB, acting on its upstream IKK kinase complex (Gil et al., 1999, 2000). Activated IKKs lead to the phosphorylation of IκB, thereby promoting translocation of activated NFκB into the nucleus. Through the regulation of both transcriptional and translational pathways, PKR is able to regulate cellular apoptosis, growth and differentiation.

I.6.2 2′-5′ oligoadenylate synthetases (2′5′OAS)

Similar to PKR, 2′5′OAS are IFN-inducible proteins that act in a dsRNA-dependent manner (Zhou et al., 1993). There are three members in the OAS family including OAS1, OAS2 and OAS3. These proteins contain a conserved domain known as the 2′-5′OAS unit that corresponds to the first 346 amino acids (Hovnanian et al., 1998). Though OAS proteins lack the classical binding site for dsRNA, data from crystal structures and mutagenesis studies suggest that there is a conserved motif for ligand recognition (Hartmann et al., 2003; Rebouillat et al., 1999). In the presence of dsRNA, 2′5′OAS is activated and begins to synthesize 2′-5′ oligonucleotides of adenylate (2-5A) of various sizes, dependent on the specific OAS. 2-5A is a ligand for RNase L, a riboendonuclease comprised of nine ankyrin repeats, a number of protein-kinase like motifs and an RNase domain (Zhou et al., 1993). Binding of 2-5A transforms the inactive monomeric RNase L into its activated dimeric conformation, which functions to cleave single-stranded RNA at the 3′ side of the UpAp or UpUp regions (Dong and Silverman, 1995; Dong et al., 1994; Tanaka et al., 2004). This process antagonizes viral replication: (1) cleavage of viral genomic RNA (Li et al., 1998), (2) degradation of viral mRNA, (3), because RNase L cleaves both viral and host mRNA species, the availability of host
proteins required for viral replication will be affected (Banerjee et al., 2000; Smith et al., 2005). In addition, cleavage of single-strand RNA can generate short dsRNAs that can serve as ligands for cytosolic sensors like RIG-I and MDA5 (Malathi et al., 2007). Activation of these cytosolic PRRs will lead to the additional production of type I IFNs and enhance antiviral responses. Lastly, RNase L activation has also been associated with the induction of apoptosis through cytochrome c and caspase 3-mediated events, invoking another mechanism for inhibiting viral replication (Castelli et al., 1997; Rusch et al., 2000).

I.6.3 The Mx proteins

Orthomyxovirus resistance (Mx) proteins were originally identified as IFN-sensitive factors in a mouse strain (A2G) that exhibited higher resistance to influenza virus infection compared to other mouse strains (Horisberger et al., 1983; Lindenmann, 1964). Members of the Mx family belong to the large GTPase family (Staeheli et al., 1993) and contain a GTPase domain at the N-terminus, a central interactive domain (CID), and a leucine zipper (LZ) motif at the C-terminus (Haller et al., 2007). The cellular localization of Mx proteins varies among the different isoforms and is also species-dependent. The mouse Mx1 protein resides in the nucleus, as a result of a nuclear localization signal (NLS) in its C-terminus, and its mechanism of antiviral action differs from cytoplasmic Mx proteins such as MxA (human) and Mx2 (rodent) (Staeheli and Haller, 1985; Staeheli et al., 1986; Staeheli et al., 1993). Inside the nucleus, Mx1 resides in a subnuclear partition known as the promyelocytic leukemia protein nuclear body (PML NB) or also known as nuclear domain 10 (ND10), and interacts with molecules
such as Sp100, Daxx and factors of small ubiquitin-related modifier-1 (SUMO-1) (Chelbi-Alix et al., 1995; Engelhardt et al., 2001; Trost et al., 2000). The precise antiviral mechanism of action of Mx1 during viral infection remains unknown. Interestingly, experiments with Thogotovirus (THOV), a member of the orthomyxovirus family, suggest that the cytoplasmic MxA protein can sequester viral nucleocapsids to prevent their translocation into the nucleus, where viral replication and virion pre-assembly takes place (Kochs and Haller, 1999a, b). This activity does not seem to require the GTPase activity of MxA, but the oligomerization of MxA seems to be important for both ligand recognition and protein stability (Haller and Kochs, 2002).

Distinct from those described above, other IFN-inducible proteins have been shown to participate in mediating type I IFNs responses, e.g. TRAIL, viperin, p21, p200 family members, caspases, though for some, their precise mechanisms of action requires ongoing investigation (Brierley and Fish, 2005b).

I.7 Biological response of type I IFNs

I.7.1 Antiviral

The signature response of type I IFNs is the induction of cellular resistance to viral infection. By modulating different cellular signaling cascades, type I IFNs can inhibit virus infection by targeting different stage(s) of viral replication. IFN-α has the ability to inhibit the entry of VSV through secreting antiviral factor(s) that remain to be identified (Basu et al., 2006). Hepatitis virus entry is also blocked by IFNs, mediated by down-regulation of the viral cell surface receptor, SR-BII (Murao et al., 2008). The
effector functions of IFN-inducible PKR, Mx and 2’5’OAS/RNase L discussed above, can cooperate to inhibit viral replication and viral protein synthesis. Studies with human immunodeficiency virus (HIV) and human T-cell leukemia virus type 1 (HTLV-1) suggest IFNs can also exert their inhibitory effects at the viral assembly stage (Dianzani et al., 1998; Feng et al., 2003; Oka et al., 1990). Furthermore, IFN-inducible ISG15 expression can inhibit the budding of Ebola virus through disrupting the ubiquitination of its VP40 protein (Okumura et al., 2008).

### I.7.2 Antiproliferative effects and apoptosis

IFN-α was the first cytokine used clinically for anti-tumour therapy, based on potent antiproliferative activity. Currently, recombinant IFN-αs are used clinically for certain haematological malignancies (Kirkwood and Ernstoff, 1984). Type I IFNs downregulate the expression of oncogenes such as c-myc, and up-regulate negative cell cycle regulators including IRF1 and JUND (Jia et al., 2007; Knight et al., 1985; Papageorgiou et al., 2007). There are many ISGs that code for pro-apoptotic factors to regulate cell survival. Studies in cells of different lineages indicate that type I IFNs can up-regulate Fas (CD95), caspases, members of regulator of apoptosis family, and other factors that function to stimulate or sensitize target cells to apoptosis (Juang et al., 2004; Selleri et al., 1997). Certainly, induction of cell death is also an effective mechanism against viral infection.
I.7.3 Immunomodulation

Type I IFNs modulate functions of effector cells from both the innate and adaptive immune system (Brierley & Fish, JICR 2002). IFNs will promote the survival of memory T cells via IL-15 stimulation (Rogge et al., 1998). IFNs induce B cell maturation and influence immunoglobulin (Ig) class switching. NK cells are activated by type I IFNs to increase effector functions (Biron et al., 1999). Expressions of chemokines and chemokine receptors are also regulated by type I IFNs, allowing differential trafficking of immune effectors to sites of inflammation (Salazar-Mather et al., 2002).

I.8 Viral evasion of IFN antiviral effects

Given the critical role of type I IFNs as a first line of defence against infection, it is not surprising that many viruses have evolved ways to target this system as a means to increase their replication efficiency. Viral-mediated inhibition of type I IFNs can be generalized into three categories, including disruption of IFN induction, disruption of IFN-inducible signaling and disruption of IFN-mediated effector functions. The non-structural protein 3/4A (NS3/4A) of Hepatitis C virus (HCV) is a serine protease that can cleave both IPS-1 and TRIF. Disruption of these adaptor proteins blocks RIG-I- and TLR3-mediated IFN induction (Li et al., 2005; Meylan et al., 2005). Paramyxoviruses such as simian virus 5 (SV5), mumps virus and parainfluenza virus, all express a V protein which is capable of blocking MDA5-mediated signaling through direct physical interactions (Andrejeva et al., 2004). Furthermore, V protein inhibits IFN signaling by targeting STAT proteins for proteasome degradation (Didcock et al., 1999). The poxvirus vaccinia virus (VACV) encodes a soluble viral receptor IFNAR homologue, capable of
blocking IFN signalling (Colamonici et al., 1995). The soluble viral receptor sequesters extracellular IFN to prevent its interaction with cell surface expressed IFNAR. VACV also encodes other viral proteins that target both TLRs and their adaptors to block the activation of IRF3 and NFκB, thereby inhibiting IFN production (Bowie et al., 2000).

I.9 Influenza A virus

Influenza virus is one of the leading infectious pathogens that threaten public health. The mortality rate of annual outbreaks ranges from a quarter to half a million around the globe, and brings morbidity to 5% -15%. Infection by influenza A virus strains accounts for the majority of severe outbreaks. The severity of influenza A virus infections was well demonstrated in the devastating 1918 Spanish flu outbreak, in which an estimated 20 to 25 million lives were lost around the world (Hampton, 2004). In 1957 and 1968 there were two other influenza virus infection pandemics and between one to four million lives were lost during each outbreak (Palese, 2004). Given this history, the recent emergence of a highly virulent avian influenza A H5N1 infection in humans has raised serious public health concerns of another potential pandemic. Interestingly, the word ‘influenza’ is derived from the Italian word “influence,” when in the mid 1700s people believed celestial stars were somehow affecting people with disease (DeLacy, 1993; Heilman and La Montagne, 1990).

I.9.1 Orthomyxoviridae family

Influenza viruses A, B, C, and thogotoviruses together constitute the orthomyxoviridae family. The types of influenza viruses can be distinguished based on
the antigenic properties of their nucleoprotein (NP) and matrix protein 1 (M1) (Cheung and Poon, 2007). Influenza A can be further categorized into different subtypes based on the antigenic properties of two of its surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). There are currently 14 HA and 9 NA that are used to designate influenza A strains in the form of HxNx (Fouchier et al., 2005; Laver et al., 1984).

**I.9.2 Components of influenza A**

Influenza A virus is an enveloped virus whose genome is comprised of eight segments of negative sensed ssRNA that encode ten different proteins (Figure I.3) (Palese, 1977). The virion contains three surface proteins, including HA, NA and M2 (Cheung and Poon, 2007). The cytoplasmic side of the viral envelope associates with M1 (Ruigrok et al., 1989). The eight segments of the ssRNA genome are wrapped around NP proteins, associated with the viral RNA polymerase and non-structural protein 2 (NS2) (Lamb and Choppin, 1983; Richardson and Akkina, 1991; Yasuda et al., 1993).

**Influenza A viral RNA polymerase**

Influenza A encodes an RNA polymerase that is responsible for its transcription and replication. This RNA-dependent RNA polymerase is comprised of three subunits: PB2, PB1 and PA. PB2 is encoded by the first viral gene segment and functions to bind and cleave the 5’cap structures of the host mRNA through its endonucleolytic activity (Blaas et al., 1982; Ulmanen et al., 1983; Ulmanen et al., 1981). The cleaved oligonucleotide is utilized both as a primer for the initiation of viral transcription and as a
recognition site for translation. PB1 is encoded by the second segment, and catalyzes the RNA-dependent RNA polymerization during viral transcription and viral genome
Figure I.3 Schematic representation of influenza A virus.
replication (Biswa and Nayak, 1994; Kobayashi et al., 1996). The RNA polymerase contains conserved motifs shared by other RNA-dependent RNA polymerases, as well as domains that interact with PB2 and PA (Digard et al., 1989). The precise function of PA is not yet clear, but evidence suggests it plays a role in both transcription and endonucleolytic cleavage of the viral polymerase during viral replication (Fodor et al., 2002; Zurcher et al., 1996).

**Hemagglutinin (HA)**

HA is an integral protein expressed on the surface of the influenza virion. It is responsible for viral entry, binding to the sialic acid-containing host cell receptors. Sialic acids belong to the nine carbon monosaccharide family detected on the terminus of glycolipids and glycoproteins. HA, depending on its subtype, can recognize sialic acid in either a sialic α2-3 galactose linkage or a sialic α2-6 galactose linkage (Connor et al., 1994; Vines et al., 1998). The activity of HA requires several post-translational modifications, including cleavage of its signal peptide, glycosylation, palmitylation and trypsin-like protease mediated cleavage to generate HA1 and HA2 (Horimoto et al., 1994). HA forms a homotrimer on the virion surface with HA1 forming a head and HA2 being the stalk of the monomer (Wilson et al., 1981). Due to the low fidelity viral RNA polymerase, the influenza genome is constantly subjected to random mutations, thus leading to the generation of different HA subtypes. There are fourteen antigenically distinct HAs, designated HA 1-14, thereby affecting the nature of seasonal vaccines and a consideration for any pandemic vaccine.
**Nucleoprotein (NP)**

The influenza NP is a 56kD protein that is abundantly expressed in the virion. It possesses an N-terminal domain that interacts with viral RNA, motifs required for oligomerization, and domains that interact with the viral RNA polymerase (Albo et al., 1995; Biswas et al., 1998; Kobayashi et al., 1994; Mena et al., 1999). The NP associates with vRNA and viral polymerase to form the viral ribonucleoprotein complex (vRNP) inside the virion. The NP also contains a nuclear localization signal (NLS), which may be responsible for the nuclear transport of vRNP during infection (O’Neill et al., 1995; Whittaker et al., 1996). In addition, the expression level of NP may regulate the switch between viral mRNA synthesis and viral genome replication by interacting with viral RNA polymerase (Shapiro and Krug, 1988).

**Neuraminidase (NA)**

The influenza NA is the second major surface protein of the influenza virion. It is expressed as a homotetramer and functions to cleave terminal sialic acid from glycoproteins or glycolipids (Hausmann et al., 1997). This activity is important for the budding of the virion, since the presence of sialic acid at the budding site can hinder viral egress via interactions with surface HA molecules (Palese et al., 1974). Like HA, NA is also glycosylated and subject to constant mutations and pressure from the immune system. There are currently nine NA subtypes that are antigenically distinct, designated NA 1-9.
Matrix proteins (M1 and M2)

Both M1 and M2 are encoded by segment seven in the influenza virus genome. M1 represents the most abundant viral protein found in the infectious particle. It functions as the major structural protein that encapsulates the vRNP complex inside the viral membrane (Ruigrok et al., 2000; Ye et al., 1999). There is evidence that M1 participates in several aspects of viral replication, including inhibition of viral replication, regulation of vRNP transport and viral assembly (Martin and Helenius, 1991; Watanabe et al., 1996). M2 is an integral membrane protein, generated from segment seven via alternate splicing and forms a homotetrameric complex on both the host cell and virion surface (Holsinger and Lamb, 1991; Lamb et al., 1985). The M2 tetramer forms the proton channel and can regulate the pH inside the virion (Ciampor et al., 1992). The reduction of pH via M2 is critical for the dissociation of M1 from vRNP, a process that is essential for subsequent viral transcription (Bui et al., 1996).

Nonstructural proteins (NS1 and NS2)

The last segment of the influenza genome also encodes two viral proteins as a result of alternate splicing. The colinear transcript encodes NS1, the sole viral protein that is absent in the infectious particle but expressed abundantly as a dimer early during infection (Nemeroff et al., 1995). NS1 resides predominately in the nucleus and is well known for its ability to inhibit type I IFNs responses (Garcia-Sastre et al., 1998). NS1 interacts with other host factors to modulate transcription, translation, survival and apoptosis, which will be discussed in more detail below. A splice variant of the same gene segment for NS1, NS2, is present in the infectious virion in small quantities (Ward
et al., 1995). Current evidence suggests that NS2 can stimulate viral replication via a hitherto unknown mechanism (Odagiri et al., 1994). NS2 also contains a nuclear export signal that is important for nuclear export of vRNPs at the late stage of infection (Neumann et al., 2000).

I.9.3 Influenza A replication cycle

Upon infection, influenza A virus first binds to sialic acid-containing glycoproteins on the host cell mediated by HA molecules (Figure I.4). The virus then enters the cell via clathrin-mediated endocytosis. Once inside the endosome, the reduction of pH leads to a conformational change that unmasks the fusion peptide of the HA molecule that is required for joining the viral envelope and the endosomal membrane (Ciampor et al., 1992; Skehel et al., 1982). Simultaneously, the M2 ion channel starts importing hydrogen ions into the interior of the virion, leading to the dissociation of the M1 oligomer and dissociation of the M1- viral RNA polymerase complex, thereby facilitating the release of vRNPs into the cytoplasm (Bui et al., 1996). vRNPs in turn are transported into the nucleus and primary transcription is initiated. The viral RNA polymerase starts to remove 5’cap structures from host mRNA and incorporates these onto the viral mRNA to facilitate transcription and subsequent translation (Blaas et al., 1982; Ulmanen et al., 1983; Ulmanen et al., 1981). Viral proteins produced early during infection include the NP, NS1, and the viral RNA polymerase complex (PB1, PB2 and PA) (Laine et al., 1982; Shapiro et al., 1987). These all contain an NLS and are subsequently transported back into the nucleus upon synthesis. Subsequently, NS1 will act to block host mRNA splicing and export, interacting with factors of the splicing
machinery and those of the nuclear export complexes (Qiu and Krug, 1994; Qiu et al., 1995). The retention of host mRNA has been proposed as a mechanism to increase the time during which the viral polymerase can act to remove 5′cap structures from the host mRNA. As infection progresses, depending on the expression of NP, newly synthesized viral RNA polymerase can produce additional viral mRNAs or mediate replication of the viral genome using viral mRNA as template. Interestingly, inhibition of host RNA polymerase has been shown to hinder viral replication, though the precise mechanism by which this occurs is not clear. Nonetheless, this suggests that the activity of the host RNA polymerase is involved in influenza virus replication (Engelhardt and Fodor, 2006).

As infection progresses, viral translation shifts to produce structural proteins such as HA, NA and M1 (Laine et al., 1982; Shapiro et al., 1987). HA, NA and M2 undergo several post-translational modifications and localize to the plasma membrane (Hughey et al., 1992; Jones et al., 1985; Roth et al., 1983). Meanwhile, in the nucleus NP starts to complex with viral genome segments and viral RNA polymerase to form vRNPs. vRNPs subsequently associate with M1 to initiate the pre-assembly of the virion (Martin and Helenius, 1991; Watanabe et al., 1996). NS2 has also been reported to interact with vRNPs and the M1 complex, but its role in viral assembly remains unclear. M1 proteins of pre-assembled virions in turn interact with the cytoplasmic domain of HA and NA to initiate viral budding from the cell plasma membrane (Ali et al., 2000). However, the influenza A virus particle requires cleavage of HA into HA1 and HA2 to become infectious, and this is thought to be mediated by intracellular proteases and proteases in the respiratory tract of the host (Bottcher et al., 2006; Horimoto et al., 1994).
Figure I.4 Replication of influenza A virus. The virion enters the cell via endocytosis, and subsequently fuses with the endosomal membrane to release its viral ribonucleoprotein complex into the nucleus. Once inside the nucleus, the viral genome is first transcribed to produce viral proteins that are needed to facilitate its genome replication. Finally, the pre-assembled viral genome associates with structural proteins at the cell membrane to initiate the release of virions.
1.9.4 Influenza virus genetic drift and genetic shift

Two major mechanisms contribute to the generation of novel antigenically distinct influenza virus strains: genetic drift and genetic shift (Webster et al., 1992). The former refers to genetic mutations created by the low fidelity viral RNA polymerase, resulting in substitutions, insertions and deletions in any segment of the viral genome. Each replication cycle has the potential to generate a number of variants. Although these mutations may result in defective virus and therefore be eliminated, other mutations may confer a selective advantage to the virus and therefore be retained. Genetic shift refers to the reassortment of segments of the viral genome when two or more different strains of influenza virus infect the same cell. When that happens, random incorporation of genome segments during viral assembly can lead to the generation of novel viral strains.

1.9.5 Influenza A virus host restriction

Influenza A viruses can infect a broad range of species including human, bird, pig and horse. Aquatic birds are the primary reservoir for influenza viruses, in which all of the subtypes can be found (Hinshaw et al., 1980). Generally, birds are infected in the gastrointestinal tract (GI) without producing any symptoms of disease, and virions are excreted in the feces (Webster et al., 1978). The specific avian species that is targeted by a particular influenza A virus is determined largely by the specificity of the viral HA and linkage conformation of the sialic acid of the host. The HA from human influenza A viruses preferentially binds to sialic acid $\alpha$2-6 galactose linkages, which are predominantly expressed in the upper respiratory tract. Avian and equine influenza A viruses usually contain HAs that preferentially bind to sialic acid $\alpha$2-3 galactose linkages.
(Connor et al., 1994; Vines et al., 1998). Notably, avian influenza virus H5N1 will bind sialic acid $\alpha$2-3 galactose sites in human lower respiratory tract tissues and recent data indicate that the respiratory tract in humans expresses both $\alpha$2-3 and $\alpha$2-6 linkages (Nicholls ref). Interestingly, pigs also express both sialic acid $\alpha$2-3/6 galactose linkages and can be infected by both human and avian influenza A viruses (Kundin, 1970; Pensaert et al., 1981; Scholtissek et al., 1983). Accordingly, public health organizations have raised considerable concern that the potential re-assortment between human and avian influenza virus strains may occur in pigs, and may lead to the emergence of aggressively virulent avian-derived human tropic influenza A viruses.

### 1.9.6 Influenza A virus infection: clinical symptoms and treatments

The earliest symptoms of respiratory influenza A virus infection in humans are associated with sore throat, cough, running nose and fever. Headache, chills, sweats and muscle soreness also occur. Patients infected with avian influenza H5N1 virus exhibit high fever, shortness of breath and gastro-intestinal disorders, such as diarrhoea. Infection like H5N1 progresses rapidly to cause respiratory distress syndrome, renal dysfunction and multi-organ failure, and may ultimately lead to death. Currently, there are two major classes of drugs available for treating influenza virus infections (Thanh et al., 2008). The first includes members of the adamantane family (amantadine, rimantadine), developed to inhibit the M2 ion channel to block the release of vRNP into the cytoplasm (Davies et al., 1964; Wang et al., 1993). The second family of inhibitors that include oseltamivir and zanamavir, target the NA protein and function to prevent viral budding (De Clercq, 2004; Moscona, 2005). However, as a result of continuous genetic mutation, there are emerging
an increasing number of influenza A virus isolates that exhibit resistance to these inhibitors, indicating the need for alternative antiviral therapies against influenza A virus infections (Bright et al., 2005; Bright et al., 2006).

I.10 NS1 and host innate immune responses

Similar to many other viruses that have developed mechanisms to evade an IFN response, influenza viruses have evolved to target this system. Interestingly, unlike vaccinia virus which targets several different components of the IFN system via different viral proteins, influenza A virus can effectively block various aspects of an IFN response through a single protein – NS1. NS1 derived its name from the fact that it is not present in the infectious virion but is expressed early during infection, i.e. a non-structural protein. It is a multifunctional protein that interacts with numerous host-derived molecules including nucleotides and proteins (Figure I.5).
Figure 1.5 Interaction between NS1 and host molecules. NS1 can block host mRNA processing by interfering with splicing, 3’ end processing and their export. It can also enhance viral protein translation and cell survival, and inhibit an IFN response.
I.10.1 Structure of NS1

NS1 possesses a dsRNA binding domain (1-73) at its N-terminus that is responsible for interacting with both viral and host ribonucleotides, and an effector domain that comprises the rest of NS1 (Qian et al., 1995). The effector domain contains numerous motifs that interact with a number of host proteins. NS1 is functionally active as a dimer. Structural analysis suggests that the N-terminal domain of NS1 comprises three $\alpha$-helices that form a symmetrical six-helical fold when NS1 dimerizes (Chien et al., 2004). Additional experiments revealed that the N-terminal domain interacts solely with the canonical A-form of dsRNA (Chien et al., 2004). The effector domain is made up of seven $\beta$-strands and three $\alpha$-helices that are stabilized through a series of hydrophobic interactions. This segment contains three well-characterized domains that interact with eIF4GI, the 30kD subunit of cleavage and polyadenylation specificity factor (CPSF30) and poly(A) binding protein II (PABII) (Burgui et al., 2003). Recent studies indicate that this region also interacts with the p85 subunit of PI3K and CrkL, as well as members of nuclear export complexes (Hale et al., 2008; Satterly et al., 2007).

I.10.2 Functions of NS1

I.10.2.1 NS1 inhibits intracellular sensors RIG-I and PKR

As mentioned, NS1 inhibits an IFN response by multiple mechanisms. NS1 will disrupt the induction of IFNs by inhibiting the intracellular sensor RIG-I, mediated by direct physical interactions (Guo et al., 2007). RIG-I plays a critical role in detecting ssRNA during influenza A virus infection. Its activation leads to association with the
downstream adaptor IPS-1, and eventually leads to the phosphorylation of IRF3 and subsequent transcriptional activation of IFN-β (Pichlmair et al., 2006; Yoneyama and Fujita, 2007). Both IRF3 translocation and NFκB activation have been shown to be impaired in the presence of NS1 upon dsRNA stimulation, which in turn blocks the induction of both proinflammatory cytokines and IFNs (Donelan et al., 2004; Wang et al., 2000). NS1 can also associate with PKR, though it remains unclear whether this interaction is dependent on the presence of dsRNA. This association blocks PKR-mediated phosphorylation of eIF2α and prevents translation arrest in the host during viral infection (Lu et al., 1995).

I.10.2.2 NS1 inhibits host mRNA processing and export

Inside the nucleus, NS1 functions to disrupt post-transcriptional processing of host mRNAs at multiple stages, including splicing, polyadenylation and nuclear export. Pre-mRNA splicing is carried out by the spliceosome, which involves a series of base pair interactions between the transcript and a series of small nuclear RNAs (snRNAs) including U6 snRNA (Berget and Robberson, 1986; Pironcheva et al., 1988). NS1 can associate with U6 snRNA via an interaction between its dsRNA binding domain and the stem loop region of U6 snRNA (Qiu et al., 1995). Binding of U6 snRNA by NS1 blocks the subsequent interactions with other snRNAs and disrupts the catalysis of pre-mRNA splicing. Studies have shown that transcription of the IFN-β gene is not affected by NS1, but that there is an accumulation of its pre-mRNA transcript in the presence of NS1 (Qiu et al., 1995). Aside from disrupting splicing, the effector domain of NS1 can also block the polyadenylation of host mRNA, via interactions with CPSF30 and PABII (Burgui et
al., 2003; Chen et al., 1999; Nemeroff et al., 1998). The generation of mature host mRNA involves CPSF-mediated endonucleolytic cleavage of the 3’ end followed by addition of a series of adenylates. This 3’ end processing is required for mRNA export and prolongs mRNA half-life from the activity of endonucleases (Wang and Kiledjian, 2000). Following 3’ cleavage, PABII functions to catalyze the elongation of the polyadenylate tail. By binding to CPSF and PABII, NS1 suppresses the generation of mature host mRNAs. Recently, NS1 has been shown to form inhibitory complexes with the mRNA export machinery, including NXF1/TAP, p15/NXT, Rae1/mrnp41 and E1B-AP5 (Satterly et al., 2007). Though the functional consequences of these interactions remains unclear, the outcome is to block host mRNA export during infection. NS1 inhibits the expression of a number of genes including pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL18 and macrophage inflammatory protein-1 alpha (MIP1α), and as well as ISGs such as the IFNαs, PKR, and 2’5’OAS (Stasakova et al., 2005). However, gene expression microarray analysis and other studies have indicated that inhibition of host gene expression by NS1 is selective and does not affect the export of viral mRNAs. This selectivity may relate to the ability of NS1 to recognize the 5’ untranslated region (UTR) of viral mRNA (Chen and Krug, 2000).

I.10.2.3 NS1 stimulates viral protein translation

Aside from its ability to inhibit the induction of IFNs-α/β, NS1 can modulate other cellular pathways during infection to support viral replication. NS1 can interact with the translation initiation factor eIF4GI of the translational machinery via its effector domain. This interaction in conjunction with the ability of NS1 to recognize 5’ UTR of
viral mRNAs have been proposed as mechanisms by which the virus is able to specifically enhance the translation of viral proteins during infection (Enami et al., 1994; Salvatore et al., 2002).

I.10.2.4 NS1 and cell survival

*In vitro*, NS1 expression can induce apoptosis of a number of cell types, including Madin-Darby Canine Kidney (MDCK) and lung epithelial carcinoma A549 (Schultz-Cherry et al., 2001; Zhang et al., 2007) cells. Caspases and p53 activation are elevated in the presence of NS1. Interestingly, recent studies revealed that NS1 can activate PI3K via an interaction with the regulatory subunit of PI3K, p85, through a putative SH2-binding domain. Activation of PI3K by NS1 in turn leads to the downstream activation of Akt (Ehrhardt et al., 2007; Shin et al., 2007). Activated Akt can regulate numerous cellular processes including cell survival. Indeed, the presence of NS1 has been shown to delay cell apoptosis during infection (Ehrhardt et al., 2007). Recent evidence suggests influenza A virus infection may up-regulate Akt activity during the early phase of infection, to prevent rapid host cell death, then initiate apoptosis at later stages of infection (Zhirnov and Klenk, 2007).

I.10.3 NS1 and virulence

The emergence of a highly pathogenic avian H5N1 influenza A virus in Hong Kong has resulted in considerable activity to determine the viral factors that contribute to its virulence. Using recombinant viruses, there are data that indicate that the viral proteins HA, PB2 and NS1 can influence the degree of virulence of influenza A (Jackson et al.,
NS1 from highly virulent strains of influenza A has been shown to be more effective at inhibiting a type I IFN response when compared to other less virulent influenza A viruses. Virus strains without or with a defective NS1 cannot propagate in cells with an intact type I IFN system, again highlighting the importance of IFNs in host defence (Garcia-Sastre et al., 1998). Like other influenza virus proteins, there are many variants of NS1 as a result of mutations, with several critical amino acid residues that have been shown to correlate with virulence in the host. Glutamic acid substitution of aspartic acid at position 92 in NS1 has been described in swine models to confer resistance to various cytokines including TNF-α and IFN-α (Li and Wang, 2007; Seo et al., 2004). Other studies indicate that an NS1 with a five amino acid deletion at positions 80-84, confers enhanced virulence to influenza A viruses, though the molecular basis of this remains to be determined (Long et al., 2008).
I.11 Thesis hypothesis and objectives

**Hypothesis:** Influenza virus H5N1 NS1 interferes with IFN-α/β inducible signal transduction.

**Objectives:** To investigate the effects of NS1 on different aspects of type I IFN signaling and evaluate the therapeutic potential of type I IFNs against H5N1 influenza A infection.
CHAPTER II
MATERIALS AND METHODS

II.1 Cells, viruses and reagents

The human cervical carcinoma cell line HeLa was obtained from ATCC (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen). Plasmid pBudCE4.1-H5N1 NS1-HA (A/Duck/Hubei/L-1) were kindly provided by Dr. Bing Sun from Shanghai Pasteur Institute. Fresh lung biopsies were obtained from patients having surgical resection of lung tissue in Queen Mary Hospital, all of whom gave informed consent under a study approved by The Hong Kong University and Hospital Authority (Hong Kong West) Institutional Review Board. The biopsies or tissue fragments were excess to the requirements of clinical diagnosis. Non-tumor lung tissue from each donor was cut into multiple fragments (2-3mm). The tissues were immediately placed into culture medium (F-12K nutrient mixture with L-glutamine, and antibiotics) and infected with influenza A H5N1 (A/Vietnam/3046/04) viruses within three hours of collection. Influenza virus was used at a titer of $1 \times 10^6$ 50% tissue culture infectious doses (TCID$_{50}$)/ml. Biopsies with no viruses were used as controls. The biopsy or tissue fragments were incubated at 37 °C for 18 h. The DNase-treated mRNA from infected ex vivo organ culture was extracted using RNeasy mini kit (Qiagen Hilden, Germany). The cDNA as synthesized from cDNA using oligo-dT primers and Superscript III reverse transcriptase (invitrogen). The infection of primary human lung tissue with influenza A virus were performed by Dr. John Nicolls laboratory (University of
Human recombinant IFN alphacon-1 (IFN alphacon-1, specific activity, \(6 \times 10^8\) U/mL) was provided by Intermune (Brisbane, CA). Human IFN-\(\beta\) (specific activity, \(1.2 \times 10^7\) U/mL) was generously provided by Darren Baker (Biogen Inc., MA). Antibodies against p-STAT1, p-STAT2, p-STAT3, STAT1, STAT3, HA, tubulin, SOCS1 and SOCS3 were purchased from Cell signaling. Antibodies against STAT2, Histone H1 and \(\beta\)-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary HRP-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were purchased from GE healthcare Limited (UK).

II.2 Transfection and virus infection

Cells \((2 \times 10^5)\) were transfected using Lipofectamine LTX (Invitrogen, CA) according to manufacturer protocol. Briefly, cells were seeded in 6 well plates 24 hours before transfection. Plasmid DNA and transfection reagent were mixed in serum-free media and incubated for 30 minutes at room temperature. Tranfection complexes were then added gently to the 6 well plate. For H5N1 influenza A (A/Vietnam/3046/04) infection, primary lung tissues were first pre-treated with either PBS or IFN alphacon-1 \((1 \times 10^4\) U/mL) for 24 hours, tissue were then incubated with virus \((1 \times 10^6\) TCID/mL) in 24 well plate for 30 minutes, tissues were subsequently washed twice with PBS and placed in F12K medium for 18 hours. The infection of primary human lung tissue with influenza A virus were performed by Dr. John Nicolls laboratory (University of HongKong, China)
II.2 Immunoblotting and immunoprecipitation

Cells were lysed in 50 µl of lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride). Protein concentration in lysates was determined using the Bio-Rad protein DC assay kit (BioRad laboratories, Hercules, CA). 40 µg of protein lysate/sample was denatured in 5x sample buffer and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a nitrocellulose membrane, followed by blocking with 5% bovine serum albumin (w/v) in TBS-T for 1 h at room temperature. Membranes were probed with the specified antibodies overnight. Membrane was washed three times (5 minute/each time) with 1X TBS-T buffer. Membrane was then probed with appropriate secondary antibodies. Proteins were visualized using the ECL detection system (Pierce, Rockford, IL). For immunoprecipitations, cells transfected with vector alone or H5N1 NS1 plasmid were incubated in hypotonic lysis buffer containing 10 mM HEPES, pH 7.4 for 30 minutes on ice and the suspension was then briefly sonicated. The suspension was incubated for 30 minutes at 4°C, followed by centrifugation at 14 000 rpm for 30 min at 4°C. The supernatant was collected and protein concentration was measured using the Bio-Rad protein assay kit. 500µg of proteins were incubated with 1µg of either anti-STAT1 antibody or anti-STAT2 antibody or IgG isotype control antibody. Immune complexes were immunoprecipitated with protein A/G-sepharose beads (Santa Cruz Biotechnology) and washed 6 times with HEPES buffer. Beads were then denatured in 5x sample reducing buffer and resolved by SDS-PAGE.
II.3 Cell sorting and flow cytometric analysis

24 hours post transfection, cells were first washed with PBS and subsequently incubated with versene for 15 minutes. Suspended cells were collected and centrifuged at 1500 rpm. Cell pellets were then resuspended at $1 \times 10^6$ cells per 100$\mu$L of 2% FCS/PBS. $5 \times 10^5$ GFP$^+$ sorted cells were incubated with either anti-human IFNAR1 (Biogen) or anti-human IFNAR2 antibody (Caltag), followed by PE-conjugated anti-mouse IgG antibody. As isotype controls, cells were incubated with PE-labeled isotype control IgG antibody (eBioscience). All analyses were performed using the FACSCalibur and CellQuest software. Cells were gated based on forward and side scatter.

II.4 RNA extraction and cDNA synthesis

Cells were lysed using buffer RLT+β mercaptoethanol with Qiagen QIA-shreddar columns. RNA isolations were carried out using Qiagen RNeasy mini kit according to the manufacturer's protocol, including on column DNA digestion. Total cellular RNA was eluted in RNase-free water. The concentration of RNA was subsequently determined by UV spectrophotometry at 260nm wavelength (Beckman). cDNAs were synthesized using 0.5$\mu$g RNA according to the manufacturer’s protocol (Invitrogen). cDNA from primary human lung tissue were provided by Dr. John Nicolls laboratory (University of HongKong, China)

II.5 Real time-polymerase chain reaction (RT-PCR)

Real-time PCR were performed using a LightCycler® (Roche) in conjunction with LightCycler® FastStart DNA Master SYBR Green PLUS I Kits (Roche). Reactions
were performed in 20 µL containing 4µL Master SYBR GreenPLUS buffer at a final concentration of 1X, 5µL of 0.1µg/µL cDNA. 9µL of PCR grade water and 1µL of each 20 µM forward and reverse primers. PCR reactions were performed under the following conditions: pre-incubation at 95°C for 10 minutes, followed by 45 amplification cycles of denaturation for 10 seconds, annealing for 5 seconds, extension at 72°C for 10 seconds, melting curve analysis at 65°C for 15 seconds and a continuous acquisition mode of 95°C with temperature transition rate of 0.1 per second. The data were subsequently analysed using software RealQuant. Primers for IFN-α2 (PPH00379A-200) and IFN-β (PPH00384E-200) were purchased from SA Bioscience (Frederick, MD). Other PCRs were performed using the following primers:

**IFNAR1**
(forward) 5’ CACTGACTGTATATTGTGTGAAAGCCAGAG 3’
(reverse) 5’ CATCTATACTGGAAGAAGGTATTAAAGTGATG 3’

**IFNAR2**
(forward) 5’ ATTTCCGGTGTCATCTTTATCAT 3’
(reverse) 5’ACTGAACAACGTTGTGTTCC 3’

**Influenza A M gene**
(forward) 5’ CTTCTAAACCAGGTCGAAACG 3’
(reverse) 5’GGCATTTTGGACAAAGCGTCTA 3’

**ISG15**
(forward) 5’ TCCTGGTGAGGAATAAACAAGG 3’
(reverse) 5’ CTCAGCCAGAACAGGTGTC 3’
PKR  
(forward) 5’ GCCTTTTCATCCAAATGGAATTC 3’  
(reverse) 5’ GAAATCTGTTCGCTTGCTCATG 3’  

2’5’OAS  
(forward) 5’ AGCTTCATGGAGAGGGGCA 3’  
(reverse) 5’ AGGCCTGGCTGAATTACCCAT 3’  

β-actin  
(forward) 5’ACATGGAGAAAAATCTGGCAC 3’  
(reverse) 5’ GTAGCAGCTTCTCCTTAATGT 3’  

II.6 Electrophoretic mobility shift assay  
10µg of nuclear protein from untreated or IFN-treated cells were extracted as described previously (Brierley et al., 2006). Extracts were incubated with 1µg poly(dI-dC)poly(dI-dC) for ten minutes at 4°C in buffer containing 60mM EGTA, and 5% Ficoll (final volume 30µl). 40,000 counts per minute (cpm) of radiolabeled SIE (5’- AGCTTCATTCCCGTAAATCCCT) were added and the reaction mixture was incubated for an additional 20 minutes at ambient temperature. Protein-DNA complexes were resolved on a 4.5% polyacrylamide gel using 0.5X TBE (final concentration 45mM Tris borate, 1mM EDTA) as running buffer. Gels were dried and exposed to autoradiographic film (Kodak BioMax MS) overnight at -80°C.
II.7 Immunohistochemistry and Confocal microscopy

Cells transfected with H5N1 NS1 plasmid were stained as described previously (Rahbar et al., 2006). Various proteins were visualized using fluorescence-conjugated secondary antibodies (Alexafluor-488: green, Alexafluor-555: red and Alexafluor 647: blue) (Amersham Biosciences, Cardiff, United Kingdom). Images were collected using an upright Leica SP2 confocal laser-scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany), a 100x oil immersion lens (1.4 numerical aperture), and a x4 digital zoom. Laser excitations were 488 nm (Ar/Kr) and 543 nm (He/Ne), attenuated to 10% and 50%, respectively, by way of an acoustic-optical transmission filter. Sequential scan mode was used to eliminate cross talk of detected signals, which were filtered between 500 to 530 nm and 560 to 660 nm. Image resolution was 512 dpi by 512 dpi (12 bit), and line averaging (4x) was used. Optical sections were collected at 0.5µm intervals through the entire cell.
CHAPTER III

RESULTS

III.1 Influenza virus H5N1 NS1 localizes primarily in the nucleus of HeLa cells

In a first series of experiments, plasmid encoding HA-tagged H5N1 NS1 was introduced by transfection into HeLa cells. Cells were fixed and stained at 8 and 24 hrs post transfection, and analyzed for NS1 expression using a fluorescent conjugated anti-HA antibody and immunofluorescence microscopy. Expression of H5N1 NS1 was observed at 8 hrs post transfection, and the data reveal that NS1 localized predominantly in the nucleus, detectable in the cytoplasm at 24 hrs (Figure III.1).
**Figure III.1** H5N1 NS1 localizes predominantly in the nucleus of HeLa cells. HeLa cells were transfected with HA-tagged H5N1 NS1 plasmid. HeLa cells were fixed and stained for HA (Red) at different time points post transfection and analyzed using immunofluorescence microscopy. Data are representative of two independent experiments.
Time post transfection (hours)
III.2 H5N1 NS1 expression inhibits both IFN-inducible STAT phosphorylation and nuclear translocation

To determine the effect of H5N1 NS1 protein expression on type I IFN signaling, HeLa cells were transfected with either vector alone or plasmid containing the H5N1 NS1. 24 hours post transfection, cells were serum starved, then either left untreated or treated with IFN-β (1×10³ U/mL) for 15 minutes. Protein extracts were collected and the IFN-inducible activation of STAT proteins was analyzed by immunoblotting. In contrast to cells transfected with vector alone, there was a notable reduction in IFN-inducible STAT1, STAT2 and STAT3 phosphorylation in cells expressing H5N1 NS1 (Figure III.2A). To examine the effect of NS1 on IFN alfacon-1 inducible STAT phosphorylation, nuclear and cytoplasmic fractions were isolated post IFN alfacon-1 (1×10³ U/mL) stimulation and analyzed by immunoblot. In comparison to vector transfected cells, the amount of phosphorylated STAT proteins was reduced in the presence of H5N1 NS1 protein, though the extent of reduction was less when compared to IFN-β treatment (Figure III.2B). Furthermore, to confirm that this inhibition of IFN-inducible STAT phosphorylation occurred only in cells expressing NS1, in an identical series of transfection experiments cells were fixed and stained with both anti-phospho-STAT2 and anti-HA (NS1) antibodies. In contrast to cells that lack H5N1 NS1 expression, which that exhibit strong IFN-inducible phospho-STAT2 staining in the nucleus, we identified a notable reduction in IFN-inducible phospho-STAT2 staining in H5N1 NS1-expressing cells following IFN-β stimulation (Figure III.2C).
Figure III.2 H5N1 NS1 expression inhibits IFN-inducible STAT phosphorylation. A) HeLa cells transfected with vector alone (■) or HA-tagged NS1 plasmid (●) were left untreated (−) or treated (+) with IFN-β (1×10³ U/mL) for 15 minutes, 24 hours post transfection. Cells harvested, and lysates were resolved by SDS-PAGE and immunoblotted with the indicated anti-phospho-STAT1, anti-phospho-STAT2, anti-phospho-STAT3 and anti-HA(NS1) antibodies. Membranes were also stripped and reprobed with anti-tubulin, anti-STAT1, anti-STAT2 and anti-STAT3 as loading controls. Relative fold induction of phosphorylated STAT proteins were calculated using signal intensity of phospho-STATs over total STATs and normalized with untreated, vector transfected cells. Data are representative of three independent experiments. B) HeLa cells transfected with vector alone (■) or HA-tagged NS1 plasmid (●) were left untreated (−) or treated (+) with IFN-β (1×10³ U/mL) for 15 minutes 24 hours post transfection. Cytoplasmic and nuclear fractions were purified and resolved by SDS-PAGE, then immunoblotted with the indicated anti-phospho-STAT1, anti-phospho-STAT2 and anti-phospho-STAT3 antibodies. Membrane were also probed with anti-HA antibody to confirm expression of NS1 protein, and anti-β-actin and anti-histone H1 were used as loading controls for cytoplasmic fraction and nuclear fraction, respectively. Relative fold induction of phosphorylated STAT proteins was calculated using signal intensity over loading and normalized with untreated, vector transfected cells. Data are representative of two independent experiments. C) H5N1 NS1 expression inhibits IFN-inducible nuclear translocation of phosphorylated STAT2. HeLa cells transfected with HA-tagged NS1 plasmid were treated with IFN-β (1×10³ U/mL) for 15 minutes. Cells were then fixed and stained for HA (red) and phospho-STAT2 (blue), and analyzed by confocal microscopy as described in Materials and Methods. Data are representative of two independent experiments.
C

NS1  p-STAT2  Merge
III.3 H5N1 NS1 protein expression inhibits IFN-inducible STAT:sis-Inducible Element (SIE) complex formation

STAT proteins, once activated by phosphorylation, will form functional homo- or hetero-dimeric complexes that migrate to the nucleus and target specific promoter elements to initiate transcriptional activation of ISGs. To examine the functional consequence of H5N1 NS1-mediated inhibition of IFN-inducible STAT phosphorylation, electrophoretic mobility shift assay (EMSA) studies were carried out. Specifically, HeLa cells transfected with empty vector or vector containing NS1, were either left untreated or treated with IFN-β ($1 \times 10^3$ U/mL) for 15 mins, 24 hours post transfection. Nuclear extracts were prepared, incubated with radiolabeled sis-inducible elements (SIE), then resolved by agarose gel electrophoresis to determine the formation of IFN-inducible STAT:SIE complexes. In contrast to cells transfected with empty vector, we observed a notable reduction in STAT3:3:SIE, STAT1:1:SIE and STAT1:3:SIE complexes in the presence of H5N1 NS1 protein (Figure III.3).
**Figure III.3** H5N1 NS1 expression inhibits IFN-inducible STAT:SIE complexes formation. HeLa cells transfected with either vector alone or HA-tagged NS1 plasmid were left untreated (-) or treated (+) with IFN-β (1×10³U/mL) for 15 minutes 24 hours post transfection. Nuclear extracts were isolated and incubated with ³²P-labeled SIE probe. Complexes were resolved by native gel electrophoresis and visualized by autoradiography. Data are representative of two independent experiments.
III.4 H5N1 NS1 expression leads to a reduction in IFNAR1 but not IFNAR2 cell surface expression

The inhibition of IFN-inducible STAT phosphorylation prompted us to investigate the influence of NS1 expression on upstream molecules involved in type I IFNs signaling, starting with the receptors, IFNAR1 and IFNAR2. HeLa cells transfected with vector-GFP or NS1-GFP plasmid were sorted 24 hours post transfection and analyzed for surface IFNAR1 and IFNAR2 expression using anti-IFNAR1 and anti-IFNAR2 antibodies and flow cytometry. Surprisingly, cells expressing H5N1 NS1 exhibit reduced level of surface IFNAR1 when compared to cells expressing vector alone (Figure III.4A). Notably, IFNAR2 expression was not affected by the expression of NS1. In a subsequent series of experiments we confirmed that this reduction in cell surface IFNAR1 expression was restricted to cells expressing NS1 (Figure III.4B).

To determine whether the differential surface expression of IFNAR1 and IFNAR2 in the presence of H5N1 NS1 is a consequence of regulation at the mRNA level, RNA was extracted from HeLa cells transfected with either vector-GFP or NS1-GFP plasmid 24 hours following transfection. IFNAR1 and IFNAR2 gene expression was analyzed using real-time PCR. In contrast to vector-GFP transfected cells, we observed a reduction in IFNAR1 but not IFNAR2 gene expression in cells transfected with H5N1 NS1 (Figure III.5A).

To determine if this selective inhibition occurs in the context of H5N1 influenza A infection, mRNA from primary human lung cells that were either mock infected (PBS) or infected with H5N1 influenza A virus were collected and analyzed using real-time PCR. Briefly, primary human non-tumor lung tissue obtained from patients undergoing
lung resection were isolated and infected with influenza A H5N1 virus, as described in Materials & Methods. At 18hrs post-infection, RNA were extracted from the lung tissues and used to generate cDNA. Infection with influenza A H5N1 virus resulted in a selective reduction in IFNAR1 but not IFNAR2 gene expression when compared to controls (Figure III.5B).
**Figure III.4** Expression of H5N1 NS1 reduces surface IFNAR1 but not IFNAR2 expression. **A)** HeLa cells were transfected with either GFP vector alone (green) or GFP vector containing HA-tagged NS1 gene (red). 24 hours post transfection, GFP⁺ cells and GFP’NS1⁺ cells were sorted by flow cytometry 24 hours, and their surface IFNAR1 or IFNAR2 expression were analyzed by flow cytometry. Data are representative of three independent experiments. **B)** HeLa cells transfected with HA-tagged NS1 plasmid were fixed and stained for HA (green) and either IFNAR1 (red) or IFNAR2 (red) 24 hours post transfection, then subsequently analyzed by confocal microscopy. Data are representative of two independent experiments.
Figure III.5 A) Expression of H5N1 NS1 reduces IFNAR1 but not IFNAR2 mRNA expression. HeLa cells were transfected with either GFP vector alone (□) or GFP vector containing HA-tagged NS1 (■). GFP+ cells were sorted by flow cytometry, RNA extracted and cDNA synthesized. Gene expression for IFNAR1, IFNAR2 and β-actin was measured by real-time PCR analysis. Relative percentages of mRNA reduction were normalized using β-actin gene expression as loading control. Data are representative of two independent experiments.

B) RNA from primary human lung cells either mock infected with PBS (□) or infected with H5N1 influenza A virus (■) was harvested 18 hours post infection. Gene expression for IFNAR1, IFNAR2 and β-actin was measured by real-time PCR analysis. Relative gene expression was calculated relative to β-actin gene expression and normalized to mock infected cells. Data are representative of two independent experiments.

Note: Infection of primary human lung cells, RNA extraction, cDNA synthesis were performed by John Nicolls (University of HongKong)
III.6 IFNAR1 mRNA transcript has a longer half-life than IFNAR2 mRNA

The reduction in IFNAR1 but not IFNAR2 gene expression in the presence of H5N1 NS1 suggests that there is a selective inhibition against IFNAR1, but it is also possible that both IFNAR1 and IFNAR2 gene expression are suppressed in the presence of H5N1 NS1, and the reduction in IFNAR1 that are detected were contributed by its shorter mRNA half-life. To compare the intrinsic mRNA half-life between IFNAR1 and IFNAR2, HeLa cells were treated with α-amanitin (2µg/mL), an inhibitor of RNA polymerase II & III in a time course study. RNAs were collected at different time point post treatment and used for cDNA synthesis. PCR analysis of IFNAR1 and IFNAR2 gene expression revealed that the turnover rate of IFNAR1 transcripts was slower when compared to that of IFNAR2 transcripts, indicating that the NS1-mediated reduction of gene expression is selectively targeting IFNAR1 but not IFNAR2 (Figure III.6).
**Figure III.6** IFNAR1 mRNA does not have a shorter half-life than IFNAR2 mRNA. Hela cells were incubated with α-amanitin (2µg/mL) for various time points. Total RNA was isolated and used for cDNA synthesis. Gene expression of IFNAR1 (■), IFNAR2 (■) and 18S rRNA was analyzed by PCR. Relative percentages of mRNA expression was calculated relative to 18S rRNA and normalized to untreated controls. Data are representative of two independent experiments.
α-amanitin

IFNAR1

IFNAR2

18S rRNA

Relative gene expressions (%) vs. Time (hour)

α-amanitin

0 4 8 12 24

Time (hour)

Time (hour)

0 4 8 12 16 24 28

0 20 40 60 80 100 120

Relative gene expressions (%)
III.7 H5N1 NS1 expression induces up-regulation of SOCS1 but not SOCS3

To determine the effect of H5N1 NS1 protein expression on negative regulators of type I IFN signaling, namely SOCS1 and SOCS3, HeLa cells transfected with either vector alone or plasmid containing the H5N1 NS1 gene were lysed 24 hours post transfection. Protein extracts were collected, and SOCS1 and SOCS3 expression were analyzed by Western immunoblotting. In contrast to cells transfected with vector alone, we observed an increase in SOCS1 but not SOCS3 expression in cells expressing H5N1 NS1 (Figure III.7).
**Figure III.7** Expression of H5N1 NS1 increases SOCS1 but not SOCS3 expression. HeLa cells transfected with vector alone or HA-tagged NS1 plasmid. 24 hours post transfection, cells were harvested and lysates were resolved by SDS-PAGE, then immunoblotted with the indicated anti-SOCS1 and anti-SOCS3 antibodies. Membranes were probed with anti-HA antibody to confirm expression of NS1, and anti-tubulin for loading. Relative fold induction of proteins were calculated using signal intensity over loading and normalized with vector transfected cells (SOCS1 ■, SOCS3 ■). Data are representative of two independent experiments.
III.8 IFN pretreatment up-regulates ISGS and inhibits H5N1 influenza A infection in primary human lung cells

In a final series of experiments, using non-tumor human lung tissue, we examined the effect of IFN-α, namely IFN alfacon-1, on H5N1 influenza A infection. Primary human lung tissues were collected from patients undergoing non-tumor lung biopsy at Queen Mary’s Hospital, HongKong. Tissues were either pretreated with media or IFN-alfacon-1 for 30 minutes prior to H5N1 influenza A infection. 18 hours post infection, RNA was extracted for cDNA synthesis. RT-PCR analysis of influenza A M gene expression suggests IFN-alfacon-1 pre-treatment can effectively inhibit H5N1 influenza A replication (Figure III.8A). Our analysis of various ISGs in human lung cDNA revealed that infection with H5N1 failed to invoke notable transcriptional activation of IFN-α and IFN-β (Figure III.8B). Gene expression analysis of 2’5’-OAS, PKR and ISG15, ISGs associated with an IFN-inducible antiviral response, revealed that the gene expression levels for these ISGs were not significantly altered, consistent with the failure of H5N1 infection to induce an IFN response. However, analysis of IFN-alfacon-1 treated primary human lung tissues revealed that an upregulation of expression of ISGs in both mock and H5N1 influenza A infected samples (Figure III.8B).
Figure III.8. IFN treatment inhibits H5N1 (A/Vietnam/3046/04) replication in primary human lung cells. Primary human lung cells were either left untreated or treated with IFN alfacon-1, IFN-β, or IFN-λ1 (10000 U/mL) for 30 minutes. Tissues were then infected with H5N1 influenza A virus. 18 hours post infection, RNA from cells was collected and cDNA synthesized. Expression of A) influenza M gene, B) PKR (□), ISG15 (□), 2’5’-OAS (■), IFN-α (□), IFN-β (□) and β-actin gene expression was measured by real-time PCR analysis. Data are representative of two independent experiments. Fold induction of gene expression was calculated relative to β-actin gene expression and normalized to mock infected controls.

Note: infection of primary human lung cells, RNA extraction, cDNA synthesis and M gene expression analysis were performed by John Nicolls (University of HongKong)
CHAPTER IV

DISCUSSION

The coordinated host response to virus infection involves activation of both innate and adaptive immune responses. Notably, a robust innate immune response is critical and, as described earlier, major effectors in the earliest innate immune response to infection by virus are the type I IFNs, IFN-α/β. Influenza A viruses have evolved to target type I IFNs, largely facilitated by the NS1 protein (Koehs et al., 2007). Indeed, it is likely that the IFN system is the primary target of NS1. Studies involving the use of influenza virus mutants, specifically defective NS1 mutants, demonstrated that in the absence of a functional NS1, influenza infection is significantly attenuated in cells with an intact IFN system. Conversely, replication of an influenza virus with a non-functional NS1 can be restored in cells or in mice if the IFN system is defective (Garcia-Sastre et al., 1998).

NS1 will inhibit dsRNA-mediated IRF3 phosphorylation and its translocation into the nucleus, thereby blocking the transcriptional activation of IFNs-α/β (Talon et al., 2000; Wang et al., 2000). However, the interaction between NS1 and the cytosolic sensor RIG-I is primarily responsible for the blockade of type I IFN induction during influenza infection (Guo et al., 2007; Opitz et al., 2007). Evidence from several groups indicates that RIG-I is critical in regulating type I IFN expression in response to ssRNA bearing 5’ phosphates (Hornung et al., 2006; Pichlmair et al., 2006). Knockdown of RIG-I limits IFN production during influenza infection, whereas overexpression of RIG-I can override the inhibitory function of NS1, and block viral replication.

Herein we report a novel mechanism by which the H5N1 influenza virus NS1 alters IFN-α/β signalling, possibly mediated by the selective inhibition of IFNAR1 gene
expression. The inhibitory effects of NS1 on IFNs-α/β have been largely attributed to its ability to inhibit IFN induction. Prompted by the increase of drug-resistant influenza A isolates, we initiated experiments to determine the effect of NS1 on type I IFN signaling as a way of exploring the therapeutic potential of type I IFNs during influenza A infection. Expression of the H5N1 influenza virus NS1 in HeLa cells led to the reduction of IFN-inducible STAT phosphorylation (Figure III.2). The phosphorylation-activation of STAT1 and STAT2 is critical for mediating IFN-α/β responses (Fu et al., 1990; Levy et al., 1989). In the absence of these transcriptional effector proteins, cells are unresponsive to IFNs-α/β and are highly susceptible to virus infection (Durbin et al., 1996; Park et al., 2000). IFN-inducible activation of the Jaks associated with IFNAR leads to the recruitment and tyrosine phosphorylation-activation of STATs, then their subsequent dimerization and translocation into the nucleus to activate gene expression via binding to specific elements in the promoter regions of ISGs. Examination of IFN-inducible STAT:SIE complexes, using EMSA, demonstrated a reduction in the formation of STAT1:1 and STAT1:3 complexes in the presence of the influenza virus H5N1 NS1 (Figure III.3).

A number of viruses have evolved to target STAT proteins to block the antiviral activity of IFNs. Paramyxoviruses such as SV5 and type II human parainfluenza viruses (HPIV2) block IFN signalling, mediated by their V proteins, which induce proteasomal degradation of STAT1 and STAT2 through polyubiquitination (Andrejeva et al., 2002; Precious et al., 2005). HCV core proteins block STAT1 activation and subsequent function, mediated by STAT1-core protein interactions and suppression of STAT1 gene expression (Lin et al., 2006).
Having demonstrated that the expression of the influenza virus H5N1 NS1 protein reduced the extent of IFN-inducible STAT phosphorylation and STAT-DNA binding, we undertook experiments to determine whether NS1 directly associates with STAT proteins. Immunoprecipitation studies did not reveal notable physical association between NS1 and STAT proteins, suggesting the inhibition of IFN-inducible STAT signaling was likely not a consequence of strong physical interaction between NS1 and STAT proteins. In subsequent experiments, we undertook to evaluate whether upstream effectors of the STAT proteins might be affected by NS1 expression. Flow cytometric analysis of surface IFNAR1 and IFNAR2 expression revealed a reduction in cell surface IFNAR1 in the presence of H5N1 NS1, but more interestingly, IFNAR2 surface expression remained unaltered. Immunofluorescence confocal microscopy were consistent with these observations (Figure III.4B). Based on the ability of NS1 to inhibit the generation of mature host mRNA, experiments were conducted that revealed that the reduction in surface expression of IFNAR1 was a consequence of a decrease in gene expression (Figure III.5). From these results we infer that the NS1-dependent reduction in IFNAR1 gene expression likely results in a decrease in IFN-inducible STAT phosphorylation and DNA binding. Indeed, in other studies with cardiac fibroblasts and myocytes, the higher basal levels of IFNAR1, JAK1, TYK2, IRF9 and STAT2 in the cardiac fibroblasts compared with the myocytes, correlated directly with a stronger IFN response (Zurney et al., 2007). The importance of IFNAR1 in an IFN response is further supported by earlier studies that showed that IFNAR1 null cells are non-responsive to IFN, and IFNAR1 null mice are highly susceptible to virus infections (Hwang et al., 1995). Interestingly, in clinical studies of HCV patients who did not respond or were less sensitive to IFN
therapy, evidence was provided for reduced levels of either IFNAR1 or IFNAR2 gene expression compared to IFN responders (Morita et al., 1998). Polymorphisms in the promoter region of IFNAR1 and IFNAR2 have been closely linked with susceptibility to a number of diseases including malaria, multiple sclerosis, trypanosomiasis, HCV and HIV (Aucan et al., 2003; Diop et al., 2006; Leyva et al., 2005; Tena-Tomas et al., 2007).

In contrast, overexpression of IFNAR1 and IFNAR2, as is the case in Down’s Syndrome patients, where chromosome 21 is trisomic, results in enhanced sensitivity to IFN (Mowshowitz et al., 1983). Viewed altogether, the inhibitory effect of influenza virus H5N1 NS1 on IFNAR1 gene expression is an effective mechanism to render target cells less sensitive to IFN.

In the context of viral infections, IFNs-α/β not only act as critical components of the innate immune response, but also play a prominent role in modulating the adaptive immune response. Consequently, NS1-mediated inhibition of IFN signalling will have a negative impact on the adaptive immune response. Viral clearance of influenza infection is predominantly mediated by virus-specific CD8+ cytotoxic T cells (Doherty et al., 1997). This process requires T helper 1 (Th1) cell activity, dependent on interactions with antigen presenting cells (APCs) such as dendritic cells (DC). IFNs-α/β promotes the differentiation and maturation of DCs (Santini et al., 2002; Santini et al., 2000). Incubation of blood-derived monocytes with IFN-α induces maturation/activation markers in DCs, e.g. CD83 and CD25, along with increased expression of the major histocompatibility complex (MHC), costimulatory molecules (CD80 and CD86) and chemokine receptors. Viral PAMP recognition by DCs leads to a series of downstream responses including type I IFN production that contributes to DC maturation. Mature DCs
present viral peptide-MHC complexes along with other costimulatory molecules to stimulate or prime virus-specific CD4\(^+\) and CD8\(^+\) T cells. IFNs-α/β also regulate the production of several chemokines in DCs that have a role in the recruitment of NK cells, activated or memory T cells, and pDCs to sites of infection (Lande et al., 2003; Padovan et al., 2002). Defects in any aspect of IFN signaling can lead to defective DC maturation and effector functions. Influenza A virus infection of DCs results in a block in DC maturation and subsequent ineffective T cell activation, mediated by NS1 (Fernandez-Sesma et al., 2006). NS1 expression in DCs altered the expression of a panel of genes that were required for both maturation and migration, including IFN-α and IFNAR1 (Fernandez-Sesma et al., 2006). Consistent with this finding, we observe a reduction in IFNAR1 gene expression in influenza virus H5N1 infected primary human lung cells. A similar reduction in IFNAR1 gene expression was identified in HeLa cells expressing the H5N1 NS1, suggesting the viral-induced downregulation of IFNAR1 gene expression in the infected primary lung cells was NS1-dependent (Figure III.5). Based on current knowledge of NS1, the reduction in IFNAR1 gene expression is likely mediated by inhibition of pre-mRNA splicing and/or polyadenylation. NS1 interacts with components of the splicing machinery, U6 snRNA. U6 snRNA has been shown to complex with other constituents of the spliceosome in an orderly fashion to mediate pre-mRNA splicing (Qiu et al., 1995). An association between NS1 and U6 snRNA hinders its ability to complex with other catalytic subunits of the spliceosome, thereby leading to the accumulation of pre-mRNAs in the nucleus of the host cell. Additionally, NS1 affects polyadenylation of host mRNA through targeting CPSF30 and PABII (Chen et al., 1999; Nemeroff et al., 1998). 3’ cleavage and polyadenylation of mRNAs promotes their export into the
cytoplasm, whereas mRNAs that have undergone 3’ cleavage alone are retained in the nucleus (Fuke and Ohno, 2008).

It is noteworthy that, using a distinct strategy to the NS1 protein, vaccinia virus encodes a type I IFN receptor homologue that functions to prevent IFN signaling by functioning as a decoy receptor and sequestering IFN away from cell surface receptors (Colamonici et al., 1995).

Notably, expression of H5N1 NS1 was shown to target the expression of IFNAR1 but not IFNAR2 (Figure III.5). We provide further evidence to suggest that this selective inhibition is not an indirect effect of their differential mRNA half-life (Figure III.6). The mechanism behind this selective inhibition in gene expression is currently under investigation. Though promoter analysis of IFNAR1 and IFNAR2 suggests they contain elements that can respond to type I IFNs, regulation of IFNAR1 and IFNAR2 gene expression has not been closely examined. IFNAR1 gene expression has been demonstrated to be lower in patients suffering from multiple sclerosis, and long term IFN therapy seems to increase IFNAR1 gene expression (Serana et al., 2008). In addition, HCV patients that are undergoing IFN therapy exhibit an increased gene expression for IFNAR2 when compared to naïve controls (Fujiwara et al., 2004).

Interestingly, several studies that have employed gene microarray analysis of intact or mutant NS1 transfected cells provide evidence to suggest that only a selective pool of genes are affected in the presence of intact NS1 (Fernandez-Sesma et al., 2006). Though NS1 disrupts numerous post-transcriptional modifications, reduction in gene expression does not seem to be a global effect, and the expression of many genes remain
up-regulated or unaffected in the presence of NS1 (Fernandez-Sesma et al., 2006; Shimizu and Kuroda, 2004, 2006).

Furthermore, given IFNs signaling can be also be negatively regulated by both SOCS1 and SOCS3 expression, we undertook a study to examine whether their expression levels are affected by the H5N1 NS1 (Liu et al., 2004; Song and Shuai, 1998). We provide evidence that H5N1 NS1 expression can up-regulate SOCS1, but not SOCS3, 24 hours post transfection (Figure III.7). Recent evidence from studies with H1N1 suggests that influenza infection can inhibit type I IFN signaling through up-regulation of SOCS3 expression, yet, there was no evidence for the H1N1 NS1 mediating this effect. These apparent contradictory data with the H1N1 infection data may be reflective of structural differences between the two NS1 proteins: whereas the H1N1 NS1 protein dimerizes in solution, H5N1 NS1 has distinct structural features and can form oligomers in solution (Bornholdt and Prasad, 2008).

Despite the strong inhibitory mechanism employed by H5N1, we show that exogenous IFN treatment can over-ride these effects, and effectively up-regulate a number of ISGs to establish an anti-viral state within cells (Figure III.8). Current clinical therapeutic strategies for patients with influenza A infections include the inhibitors of the Adamantine family, which inhibit the M2 ion channel to block the release of vRNP into the cytoplasm (Davies et al., 1964; Wang et al., 1993), and those that target the NA protein to prevent viral budding (De Clercq, 2004; Moscona, 2005). Despite their earlier successes, the frequency of influenza A virus isolates exhibiting resistance to these inhibitors has increased dramatically, prompting a need for alternative antiviral therapeutic strategies (Bright et al., 2005; Bright et al., 2006). Herein, we demonstrate
that IFN alfacon-1 can effectively block H5N1 influenza A replication in human lung cells, warranting further investigations into the effective use of IFNs as a viable therapeutic strategy to control H5N1 influenza A infection in humans.

The expression of IFNAR1 and IFNAR2 has not been scrutinized in the context of other strains of influenza A or NS1-expressing cells, thus it is difficult to surmise whether such selective inhibition of IFNAR1 is exclusively associated with the highly pathogenic strain like H5N1, or a conserved phenomenon across strains of influenza viruses. However, studies using recombinant virus strongly suggest NS1 plays a prominent role in dictating the virulence of influenza viruses. Substitution of NS1 derived from highly pathogenic strains such that of H5N1 or the 1918 H1N1 into the low virulence influenza viruses greatly enhances their pathogenicity in animals (Jackson et al., 2008; Li et al., 2006). Mutagenesis experiments suggest a number of key residues in NS1 are closely associated with virulence. Substitution of aspartic acid to glutamic acid at position 92 has been shown to confer resistance to antiviral cytokines like TNF-α and IFN-α in swine models of infection (Jackson et al., 2008; Li et al., 2006). Pre-treatment of cells with these antiviral cytokines did not block subsequent viral replication, and the underlying mechanism of action remains unknown. Another cluster of amino acids in NS1 that apparently contribute to virulence are five amino acids at positions 80-84. Deletion of these amino acids in NS1 has been shown to increase NS1’s effectiveness as an IFN antagonist. The H5N1 NS1 in our study was negative for the D92E mutation but it did possess the 80-84 amino acid deletion, from which we might infer that this deletion may potentially influence the reduction in IFNAR1 gene expression.
As the number of drug-resistant influenza isolates increases, much attention is being focused on NS1, in the hope of developing novel therapeutics against influenza infection (Sheu et al., 2008). Certainly, insights into the mechanism of action of NS1 as a virulence factor, as described herein, confirm that disabling NS1 is a reasonable strategy for the development of antiviral drugs targeted against influenza A viruses.
CHAPTER V

FUTURE DIRECTIONS

The inhibition of IFN-inducible STAT activation in the presence of H5N1 NS1 was examined only in the context of tyrosine phosphorylation. However, complete transcriptional activation of STAT proteins requires both phosphorylation on tyrosine as well as serine residues. Thus it would be of interest to examine the effect of H5N1 NS1 on IFN-inducible serine phosphorylation.

Another question not addressed in our studies relates to the effect of H5N1 on the gene expression ratio between different IFNAR2 isoforms. IFNAR2 has three isoforms derived from pre-mRNA splicing, with only IFNAR2c being the functional receptor that can transduce signal upon ligand binding. Ratios between functional and non-functional receptors can influence the strength and kinetics of IFN signaling. Because the flow cytometric analysis, immunofluorescence and RT-PCR studies did not allow us to distinguish among the different isoforms, subsequent experiments to examine the ratio of the different IFANR2 isoforms in the presence of H5N1 NS1 would provide insights into the effects of NS1 on IFNAR2 gene expression.

NS1 mutation experiments are planned to identify the domain(s) or amino acid residues responsible for the reduction in IFNAR1 gene expression. Several key residues have been shown to play important roles in the activity of NS1 in suppressing IFN responses, including Arg 38 and Ser 42 in the dsRNA binding domain, and Phe 103 and Met 106 in the C-terminal domain (Kochs et al., 2007). Using site-directed mutagenesis, residues 38, 42, 103 and 106 will be targeted, then the effects of expression of these
mutant NS1 constructs in HeLa cells examined in the context of IFNAR1 gene and cell surface expression.
CHAPTER VI

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