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Minireview

Influenza viruses and the NF-κB signaling pathway – towards a novel concept of antiviral therapy

Stephan Ludwig[1,*] and Oliver Planz[2]

[1] Institute of Molecular Virology (IMV), Westfaelische Wilhelms University, Von-Esmarch Str. 56, D-48161 Münster, Germany

[2] Institute of Immunology, Friedrich-Löffler-Institute (FLI), Paul-Ehrlich Str. 28, D-72076 Tübingen, Germany

* Corresponding author
e-mail: ludwigs@uni-muenster.de

Abstract

Influenza A virus remains a major public health concern, both in its annual toll in death and debilitation and its potential to cause devastating pandemics. Like any other virus, influenza A viruses are strongly dependent on cellular factors for replication. One of the hallmark signaling factors activated by viral pathogens is the transcription factor NF-κB. Activation of NF-κB leads to the up-regulation of a variety of antiviral genes. Thus, the factor is commonly regarded as a major regulator of the innate immune defense to infection. However, several recent studies indicate that influenza viruses have acquired the capability to reprogram this antiviral activity and to exploit the factor for efficient replication. These data provide novel insights into the pathophysiological function of NF-κB in the special environment of a virus-infected cell. Furthermore, the unexpected viral dependency on a cellular signaling factor may pave the path for novel antiviral approaches targeting essential cellular components rather than viral factors.

Keywords: antiviral therapy; apoptosis; caspase; influenza virus; interferon response; NF-κB; signal transduction.

Introduction

Influenza A viruses belong to the family of orthomyxoviridae and possess a genome consisting of eight single stranded RNA segments of negative polarity. The genome of approximately 13-kb encodes for up to 11 viral proteins (Lamb and Choppin, 1983; Yewdell and Garcia-Sastre, 2002). Upon attachment of the virus to cells via its surface glycoprotein hemagglutinin, the particle is taken up via endocytotic processes. Influenza viruses are among the few RNA viruses that replicate its genome in the nucleus. Thus, the virus depends on the nuclear import and export machinery of the host. Furthermore, the virus expresses a non-structural protein (NS1) that interferes with the cellular innate immune signaling to suppress antiviral responses. Since the RNA-dependent RNA polymerase complex encoded by genome segment 1–3 is very error prone, a number of genetic variants arise in every replication cycle. Thus, these viruses can easily escape selection pressures resulting from preexisting immunity or the action of antiviral agents that directly target viral components.

Survival of all viruses, including influenza viruses, is strongly dependent on a cellular host that facilitates virus replication (Ludwig et al., 1999). Depending on the biology of the virus, a variety of cellular factors are required for the invasive pathogen to complete its life cycle. Thus, cellular factors that are essential for viral replication, but at the same time may be dispensable for cell metabolism or proliferation, may be much better targets for antiviral intervention. The virus cannot easily replace these cellular functions by mutation and viral resistance should not occur. This review aims to highlight this novel concept taking the virus-activated IKK/NF-κB signaling module as an example for a suitable antiviral target inside the host cell.

The IKK/NF-κB signaling pathway

The nuclear factor κB (NF-κB) family comprises seven structurally related transcription factors that fulfill a central role in the cellular stress response and in inflammation by controlling a network of gene expression (Hayden and Ghosh, 2004). Apart from its function as regulator of the expression of inflammatory cytokines, chemokines, immunoreceptors and adhesion molecules, the factor also regulates mechanisms of controlled cell death (apoptosis) in several cell types (reviewed in Pahl, 1999; Karin and Lin, 2002). Moreover NF-κB is commonly activated upon virus infections resulting in the expression of an array of cytokine and chemokine genes (Hiscott et al., 2001).

The NF-κB family consists of a group of dimeric transcription factors that belong to the Rel family, which encompass five members: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel. Dimers containing RelA, RelB or c-Rel are transcriptional activators, whereas homodimers of p50 and p52, which are devoid of a transcription activation domain, function as repressors. Although the NF-κB subunits are ubiquitously expressed, their actions are regulated in a cell-type- and stimulus-specific manner, allowing for a diverse range of effects. Recent molecular analysis has shown that NF-κB can be induced by the so-called 'canonical' (classical) and 'non-canonical' (alternative) signaling pathways,

leading to distinct patterns in the individual NF-κB subunits that are activated and consequently lead to the induction of gene expression responses (Bonizzi and Karin, 2004). The canonical mechanism of NF-κB activation includes activation of the inhibitor of IκB-kinase (IKK) complex (Figure 1, middle panel). The IKK complex consists of at least three isozymes of IKK, named IKK1/IKKα, IKK2/IKKβ and NEMO/IKKγ. The most important isozyme for the activation of the canonical NF-κB pathway is IKK2/IKKβ that phosphorylates the inhibitor of NF-κB (IκB) and targets the protein for subsequent degradation (Karin and Ben-Neriah, 2000) (Figure 1, middle panel). This consequently leads to the release and translocation of NFκB factors p65 (also known as Rel-A; a subunit of NF-κB) and p50 (also known as NFκB1) dimers that migrate to the nucleus to exert its biological functions (Karin and Ben-Neriah, 2000; Bonizzi and Karin, 2004) (Figure 1, middle panel).

Influenza virus and the IKK/NF-κB module

NF-κB regulates expression of a variety of antiviral cytokines, including interferon (IFN) β, which is the initiator of a strong type I IFN defense program (Pahl, 1999). Thus, NF-κB has been analyzed in the context of infections with a variety of different viruses (see also Bode et al., 2008; Kieser, 2008; Wolff et al., 2008). Due to this role in antiviral gene expression, for a long time NF-κB and IKK were regarded as *bona fide* components of the innate immune response to virus infections (Chu et al., 1999). In support of that view it has been shown for influenza virus that IFNβ-promoter activity is impaired in cells expressing trans-dominant negative (dn) mutants of IKK2/IKKβ or IκBα (Wang et al., 2000; Wurzer et al., 2004). Furthermore, influenza virus titers are slightly enhanced in cells treated with an anti-IFN-α/β receptor antibody, while this is not the case in cells expressing dnIκBα. These findings indicate that influenza virus induces IFN expression via NF-κB (Wurzer et al., 2004).

It is obvious that a strong IFN expression is not beneficial for virus replication; thus, many if not all viruses have evolved strategies to evade this innate immune response. For influenza viruses, the viral non-structural protein NS1 has been identified as a major suppressor of IFNβ expression and NF-κB activation (Wang et al., 2000), introducing this protein as a prototype viral interferon antagonist (reviewed in Wolff et al., 2008). However, the antagonistic activity of NS1 is by far not exhaustive and a significant activation of IKK2/IKKβ and NF-κB as well as induction of NF-κB-dependent gene expression can still be observed in virus-infected cells or organisms

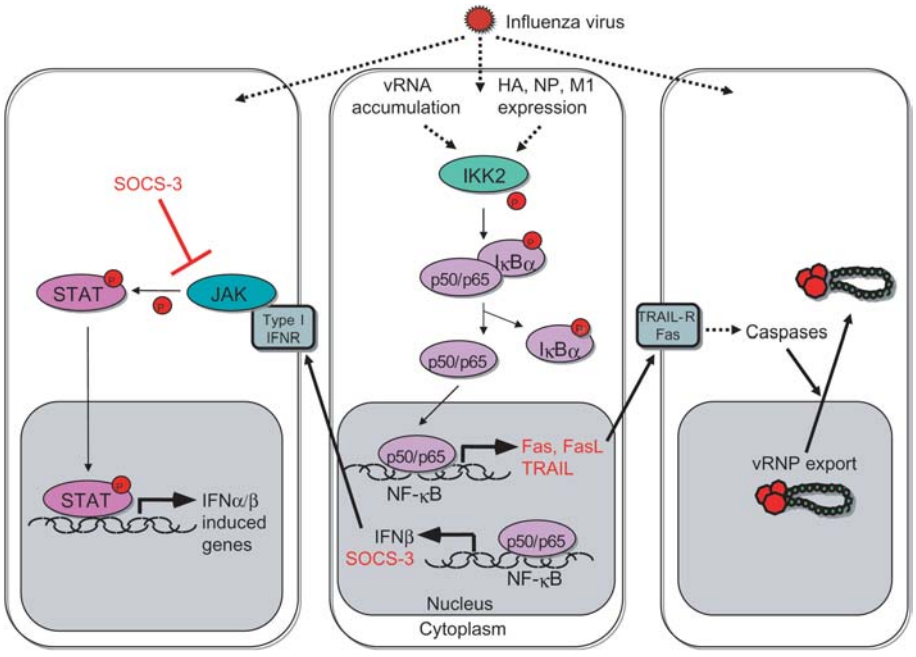

Figure 1 Two virus supportive functions of the IKK/NF-κB signaling module in influenza virus infected cells.
During productive virus infection, NF-κB regulates expression of a number of genes, including the antiviral cytokine IFNβ, the pro-apoptotic factors TRAIL, Fas and FasL and the suppressor of cytokine signaling SOCS-3. While IFNβ primarily exerts antiviral functions by inducing an innate antiviral gene expression program, the simultaneous vRNA-induced expression of SOCS-3 limits this response. Furthermore, TRAIL and FasL induce caspase activation in an auto- and paracrine fashion. Active caspases allow an enhanced release of ribonucleoprotein (vRNP) complexes from the nucleus, presumably due to caspase-mediated disruption of the nuclear pore complex. The findings suggest a scenario in which influenza virus directs antiviral NF-κB activity into an IFN-suppressive and apoptosis-promoting function. Note that some of the factors involved in the mechanisms shown, e.g., sensors for vRNA, such as RIG-I, or other transcription factors involved in type I IFN-induced signaling, such as STAT1 and 2, or IRF-9 have been omitted for the sake of clarity. Abbreviations: vRNA, viral RNA; HA, hemagglutinin; NP, nucleoprotein; M1, matrix protein; IKK2, IκB kinase 2; IκBα, inhibitor of κB α; TRAIL, TNF-related apoptosis inducing ligand; TRAIL-R, TRAIL receptor; IFNR, type I interferon receptor; STAT, signal transducers and activators of transcription; SOCS-3, suppressor of cytokine signaling-3; vRNP, viral ribonucleoprotein complexes; P, phosphate.

(reviewed in Ludwig et al., 2003, 2006). One explanation for this observation might be that influenza viral NF-κB activation is not only achieved by accumulation of viral RNA species but also by over-expression of the viral hemagglutinin, nucleoprotein or matrix proteins (Flory et al., 2000), a process that most likely cannot be blocked by the NS1 (Figure 1).

In any case, the quite high degree of NF-κB activity in influenza virus infected cells, despite the presence of a viral suppressor, have led to the hypothesis that this remaining activity may be beneficial for virus replication (reviewed in Ludwig, 2007). This challenging assumption has been recently confirmed experimentally in two independent studies demonstrating that influenza viruses replicate to a higher extent in cells with pre-activated NF-κB (Nimmerjahn et al., 2004; Wurzer et al., 2004). Remarkably, this has been shown by two completely different approaches. In one of the studies, the authors expressed a constitutively active form of IKK2 (Wurzer et al., 2004) to activate the pathway, while in the other study viral replication efficiency was explored in transformed cells with different degrees of constitutive NF-κB activity (Nimmerjahn et al., 2004). While influenza viruses replicated fairly well in Epstein-Barr virus-immortalized B-cells that exhibit a strong constitutive NF-κB activity, cells with low NF-κB activity were resistant to influenza virus propagation, but became susceptible upon activation of this pathway (Nimmerjahn et al., 2004). Conversely, progeny virus titers were reduced when grown in host cells in which NF-κB signaling was impaired either (i) by means of specific inhibitors, such as BAY11-7085 or BAY11-7082, or (ii) by the use of dn mutants of IKK2/IKKβ or IκBα (Nimmerjahn et al., 2004; Wurzer et al., 2004), or (iii) by interference with p65 expression with specific siRNAs (Figure 2). This special function of NF-κB in influenza virus infected cells appears to be different from the situation with other RNA viruses, e.g., Borna disease virus where constitutive activation of NF-κB clearly leads to a drop in virus titers most likely due to a strong induction of the type I interferon response (Bourteele et al., 2005).

From these studies, it could be concluded that influenza viruses are specifically capable of turning the antiviral activity of NF-κB into a virus-supportive action.

Redirecting NF-κB activity towards a proapoptotic function

The surprising observation of a viral dependence on NF-κB function raises the question of the underlying molecular mechanism(s). To this end, it was demonstrated that the virus supportive function of NF-κB is at least in part due to the NF-κB dependent expression of factors, such as TNF-related apoptosis inducing ligand (TRAIL) or FasL (Wurzer et al., 2004), that are known activators of a cell death program (Figure 1). Inhibition of these proapoptotic ligands by addition of soluble receptors to the supernatants of infected cells resulted in decreased caspase activity and reduced virus titers, while virus propagation was enhanced in cells that were stimulated for a short time with TRAIL and FasL (Wurzer et al., 2004). This indicates that expression of the factors is to some extent beneficial for virus replication. The action of TRAIL and FasL is executed within the cell by a family of apoptosis regulating proteases termed caspases (Thornberry and Lazebnik, 1998). Accordingly, it was shown that influenza virus propagation was also strongly impaired in the presence of either caspase inhibitors or by using siRNA to interfere with expression of a major effector caspase, caspase 3 (Wurzer et al., 2003). Caspases specifically cleave cellular proteins including those of the nuclear

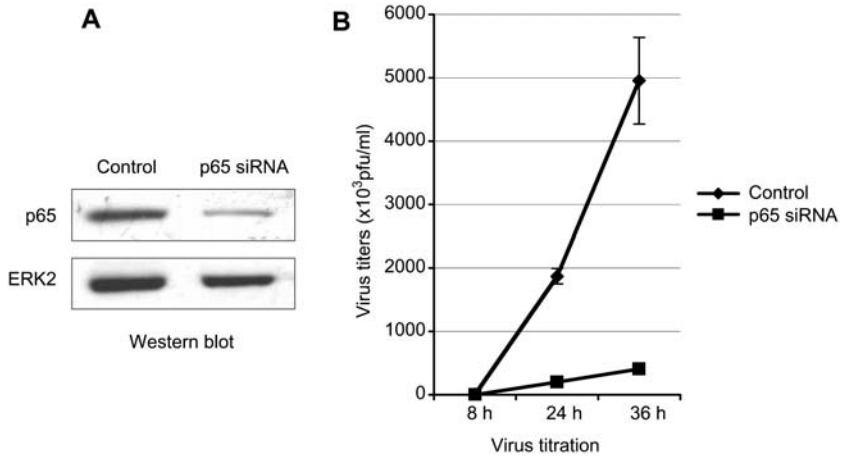

Figure 2 Inhibition of NF-κB signaling by siRNA-mediated knock-down of p65 results in reduced progeny titers of influenza A viruses.

HEK293 cells were transfected with an empty vector (control) or a vector expressing a p65-specific siRNA. (A) Cells were lysed after 24 h and expression of p65 was assessed by Western blotting. Note that p65 protein levels are significantly reduced in siRNA expressing cells. Western blots to detect expression of the MAP kinase ERK2 served as a loading control. (B) Post-transfection cells (24 h) were infected with the highly pathogenic avian influenza A virus isolate A/FPV/Bratislava/79 (H7N7) at a multiplicity of infection (MOI) of 0.01. At 8 h, 24 h and 36 h post-infection cell supernatants were removed and assayed for the number of progeny virus particles. Note that in supernatants of p65 siRNA expressing cells, the number of infectious progeny viruses is significantly reduced, while scrambled siRNA used in independent experiments did not result in altered virus titers.

pores, resulting in an enhanced diffusion limit for protein transport in and out of the nucleus (Faleiro and Lazebnik, 2000; Kramer et al., 2008). This function appears to be relevant for viral replication since in the presence of both, caspase- and NF-κB-inhibitors a nuclear retention of viral ribonucleoprotein (RNP) complexes can be observed (Wurzer et al., 2003; Mazur et al., 2007). This event most likely prevents formation of progeny virus particles (Figure 1). Interestingly, the findings are consistent with an earlier report showing that upon infection of cells that over-express the anti-apoptotic protein Bcl-2, the viral RNP complexes were retained in the nucleus (Hinshaw et al., 1994) resulting in repressed virus titers (Olsen et al., 1996).

Thus, the typical antiapoptotic function of NF-κB in response to other stimuli is converted into a proapoptotic function in the context of infection. Within an influenza virus infected cell, the activity of the NF-κB transcription factor is linked to the activation rather than inhibition of caspases that in turn support a viral nuclear export process.

While this represents one chain of events that may determine the virus-supportive function of NF-κB, recent data have revealed an additional mode of action that is likely to contribute. It could be shown that the suppressor of cytokine signaling-3 (SOCS-3) gene is strongly up-regulated in an NF-κB dependent manner due to accumulation of viral 5′triphosphate RNA in cells infected with a human H1N1 type influenza virus or with a highly pathogenic avian influenza strain of the H5N1 subtype. SOCS proteins are efficient blockers of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway (Kubo et al., 2003). This signaling pathway is also activated by type I IFN to mediate the initiation of a type I IFN induced gene expression program. Consistent with this function, the expression of SOCS-3 in influenza virus infected cells limits the antiviral gene expression responses to virus-induced type I IFN (Pauli et al., submitted). This finding provides a molecular explanation for the observation that IFN-induced gene expression is potentiated in influenza virus infected cells deficient for transcriptionally active NF-κB factors (Wei et al., 2006). The enhanced expression of IFN dependent genes may in part be due to the lack of the inhibitory action of virus-induced SOCS-3.

Taken together, these findings delineate a scenario in which influenza virus is reprogramming NF-κB action by suppression of antiviral activities and recruitment or induction of factors for virus-supportive functions, namely SOCS-mediated block of type I IFN signaling and activation of caspase-mediated RNP export from the nucleus (Figure 1).

Finally, NF-κB may not only influence pathogenesis of influenza virus by direct effects on the virus life cycle. Excessive inflammation due to overabundant production of proinflammatory cytokines and chemokines by airway epithelial cells (also known as cytokine burst) is considered an important factor in disease pathogenesis. The majority of cytokines/chemokines are regulated by NF-κB (Pahl, 1999). Consequently, activation of NF-κB in airway epithelial cells or infiltrating monocytes may strongly influence the outcome of the cytokine burst after influenza A virus infection (Bernasconi et al., 2005; Peiris, 2006).

NF-κB inhibitors as anti-influenza agents – a proof of principle

The unexpected dependence of influenza viruses on NF-κB activity raises the question whether the signaling pathway could be a suitable target for antiviral intervention. However, targeting a cellular factor raises natural concerns about unwanted side effects. First indications that at least a partial inhibition of NF-κB may be well tolerated in a host cell came from studies in which dn mutants of IKK2/IKKβ or IκBα were stably expressed in A549 lung epithelial cells or Madin Darby canine kidney epithelial cells (MDCK) (Wurzer et al., 2004). These cell lines did not show any growth disadvantage, morphological changes or enhanced death rates. Nevertheless, NF-κB inhibition led to an obvious phenotype upon an inflammatory challenge with proinflammatory cytokines or infectious agents. These data were confirmed by studies with NF-κB inhibiting agents, such as the inhibitors BAY11-7085 or BAY11-7082, that were at the same time proven to have antiviral activity (Nimmerjahn et al., 2004; Wurzer et al., 2004).

To examine the suitability of NF-κB as a target for an antiviral approach and at the same time to address the issue of drug safety, we have chosen an NF-κB inhibiting agent that is in frequent clinical use. Acetylsalicylic acid (ASA), also known as Aspirin, has been previously shown to be an efficient and quite selective inhibitor of IKK2 in low millimolar concentration ranges (Yin et al., 1998).

Given this activity, it was not surprising that ASA efficiently blocked replication of influenza viruses, including H5N1 strains, in cell culture by several orders of magnitude in a concentration range that was not toxic for the host cell (Mazur et al., 2007). Comparison to other NF-κB inhibitors or blockers of other functions that are targeted by ASA revealed that the compound indeed acts antiviral via its NF-κB inhibiting activity. As proposed earlier and according to its NF-κB inhibiting function ASA resulted in a decreased expression of TRAIL and FasL, reduced caspase activity and retention of viral RNPs in the nucleus (Mazur et al., 2007). Strikingly, ASA did not lead to the generation of resistant virus variants in multi-passaging experiments in cell culture. This was in clear contrast to the results obtained with the viral M2 ion channel blocker amantadine that resulted in a completely resistant virus population already after five consecutive passages in the presence of the drug (Mazur et al., 2007). Finally, ASA also efficiently acted antiviral *in vivo* in a mouse infection model. Application of the compound as an aerosol directly into the trachea of lethally infected mice reduced virus titers in the lung and significantly promoted survival (Mazur et al., 2007). More recent data showed that ASA as an aerosol is well tolerated by the mice and does not exhibit toxic effects (O.P., unpublished data).

In summary, these data may be taken as a first proof of principle that NF-κB inhibitors can serve as anti-influ-

enza agents *in vivo* without toxic side effects or the tendency to induce viral resistance.

Conclusion and outlook

While the IKK/NF-κB pathway also exhibits antiviral activities in an influenza virus infected cell, it seems that the pathogen has managed to redirect the activity of the transcription factor into a virus-supportive function. This function appears to rely on at least two chains of molecular events: (i) regulation of caspase-mediated RNP export, and (ii) NF-κB dependent induction of SOCS-3 that in turn inhibits type I IFN responses. Given the prominent role of NF-κB in proinflammatory gene expression, it might well be that there are still other mechanisms that contribute. It also remains to be examined whether interference with NF-κB influences the development of adaptive immune response to infection *in vivo*.

Targeting cellular factors for an antiviral strategy is still a challenging approach and the concerns about side effects are obvious. However, it should be considered that drugs targeting viral factors, such as amantadine or oseltamivir, also exhibit a wide range of side effects in patients. Thus, drug safety has to be rigorously tested in clinical trials regardless whether a drug targets a cellular or a viral factor.

Taking into account the increasing number of reports of resistance of human H1N1 influenza viruses and highly pathogenic avian H5N1 virus strains to oseltamivir, the only drug in a broader clinical use to date, it might be speculated that rapid emergence of resistant virus variants will be the fate of treatment of every drug that directly target viral factors. In that respect, the concept to target cellular factors (see also Pleschka, 2008) might be the only way to ensure that newly developed drugs will be useful and effective for a long time.

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