Short Communication

Strong interferon-inducing capacity of a highly virulent variant of influenza A virus strain PR8 with deletions in the NS1 gene

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Influenza viruses lacking the interferon (IFN)-antagonistic non-structural NS1 protein are strongly attenuated. Here, we show that mutants of a highly virulent variant of A/PR/8/34 (H1N1) carrying either a complete deletion or C-terminal truncations of NS1 were far more potent inducers of IFN in infected mice than NS1 mutants derived from standard A/PR/8/34. Efficient induction of IFN correlated with successful initial virus replication in mouse lungs, indicating that the IFN response is boosted by enhanced viral activity. As the new NS1 mutants can be handled in standard biosafety laboratories, they represent convenient novel tools for studying virus-induced IFN expression *in vivo*.

Non-structural protein NS1 of influenza A virus is a virulence factor that suppresses induction and action of the interferon (IFN) system (Garcia-Sastre, 2001; Haller *et al.*, 2006; Krug *et al.*, 2003). Deletion of NS1 attenuates viruses in IFN-competent cells and animals (Garcia-Sastre *et al.*, 1998; Kochs *et al.*, 2007b; Mordstein *et al.*, 2008).

NS1 is a multifunctional protein (Hale *et al.*, 2008; Kochs *et al.*, 2007a). Its N-terminal RNA-binding domain interferes with RIG-I-mediated induction of IFN (Donelan *et al.*, 2003; Mibayashi *et al.*, 2007; Pichlmair *et al.*, 2006). Furthermore, NS1 suppresses RNase L and PKR activation (Bergmann *et al.*, 2000; Li *et al.*, 2006; Min & Krug, 2006). Motifs in the C terminus of NS1 are involved in binding of the 30 kDa subunit of CPSF (cleavage and polyadenylation specificity factor), thereby provoking a general shut-off of cellular gene expression by blocking the processing of cellular mRNAs (Das *et al.*, 2008; Kochs *et al.*, 2007a; Noah *et al.*, 2003; Satterly *et al.*, 2007). NS1 also affects the antiviral activity of IFN by preventing the establishment of an intracellular antiviral state (Hayman *et al.*, 2006; Seo *et al.*, 2002). Independently of its effects on the IFN system, NS1 also seems to influence virus replication (Falcon *et al.*, 2004), and an association of NS1 with the viral polymerase complex has been suggested (Kuo & Krug, 2009; Marion *et al.*, 1997). By interacting with the cellular translation-initiation factor eIF4GI via its central domain (aa 74–113), NS1 is able to stimulate translation of viral transcripts (Burgui *et al.*, 2003; Enami

et al., 1994). In addition, NS1 can activate the cellular phosphatidylinositol 3-kinase/Akt pathway, thereby affecting virus replication (Ehrhardt *et al.*, 2007; Hale *et al.*, 2006; Shin *et al.*, 2007).

Influenza viruses with deletions in NS1 are potent IFN inducers. However, as these mutants are strongly attenuated, their cytokine-inducing capacity might be compromised *in vivo* (Garcia-Sastre *et al.*, 1998; Quinlivan *et al.*, 2005). Therefore, the IFN-inducing capacity of such mutant viruses *in vivo* is greatly determined by their ability to replicate in IFN-competent animals. In the present study, we addressed this issue experimentally by introducing identical NS1 mutations into two variants of influenza virus strain A/PR/8/34 (H1N1) that differ greatly in their ability to replicate in the lungs of mice (Grimm *et al.*, 2007). As we predicted based on our assumption, NS1 mutants derived from the virus variant with intrinsically enhanced replication speed induced a much more robust IFN response in infected mice than the corresponding NS1 mutants derived from standard virus.

Using a PCR-based strategy (Quinlivan *et al.*, 2005) and a newly established Madin–Darby canine kidney (MDCK) cell line that stably expresses an NS1–green fluorescent protein fusion protein, we generated a mutant of highly virulent A/PR/8/34 strain (hvPR8) in which the NS1 gene was completely deleted. The enhanced replication capacity of hvPR8 in mice is determined by its viral surface proteins and the viral polymerase (Grimm *et al.*, 2007; Rolling *et al.*,

2009). IFN induction and virulence of this mutant virus (designated hvPR8-delNS1) were compared with those of the corresponding mutant of standard A/PR/8/34 (designated msPR8-del NS1) from Mount Sinai Hospital (Garcia-Sastre *et al.*, 1998). We first infected mouse embryo fibroblasts that carry firefly luciferase under the control of the IFN-β promoter (Lienenklaus *et al.*, 2009). As expected, both wild-type viruses induced only weak expression of the reporter gene, whereas both delNS1 viruses stimulated the IFN-β promoter strongly (Fig. 1a).

Similarly, human A549 lung fibroblasts infected with the delNS1 viruses secreted at least 100-fold more IFN into the culture supernatant than cells infected with the corresponding wild-type viruses (Fig. 1b).

IFN induction depends on activation of IFN-regulatory factor 3 (IRF3), a process that can be monitored by performing IRF3-dimerization assays (Iwamura *et al.*, 2001). Accumulation of IRF3 dimers was observed in cells infected with hvPR8-delNS1, but not in cells infected with
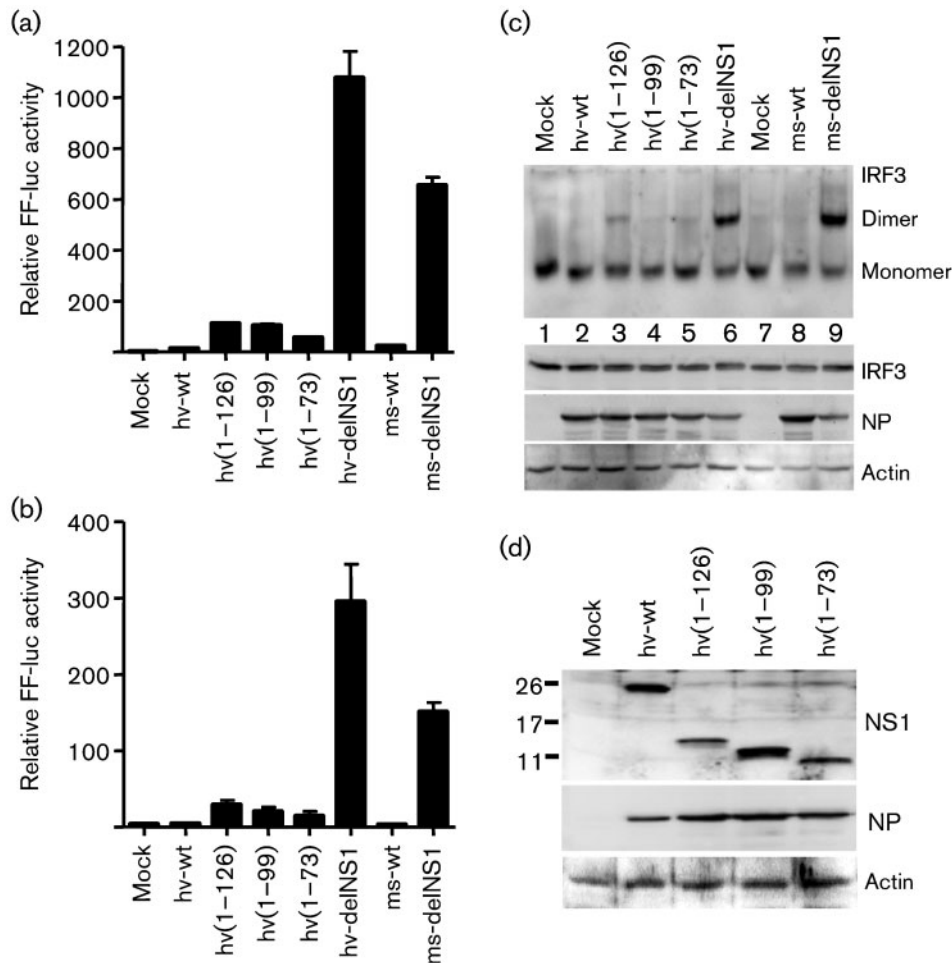

Fig. 1. IFN-inducing capacity of NS1 mutants in cell culture. (a) Activation of the IFN-β promoter in mouse embryo fibroblasts expressing firefly luciferase under the control of the IFN-β promoter. Luciferase activity was determined in lysates of cells infected at an m.o.i. of 1 for 18 h. Data from three experiments are shown; error bars indicate variations of the mean. (b) Induction of type I IFN in human A549 cells. Cells were infected (m.o.i. of 1) for 18 h and culture supernatants were then dialysed against low-pH buffer to inactivate virus, as described previously (Kochs *et al.*, 2007b). After the pH was brought back to neutral, these supernatants were added to 293T cells carrying firefly luciferase under the control of the *Mx1* promoter (Jorns *et al.*, 2006). Eighteen hours later, luciferase activity of lysates from indicator cells was determined. Data from three experiments are shown; error bars indicate variations of the mean. (c) Activation of IRF3 in 293 cells. Cells were infected (m.o.i. of 1) for 18 h and lysed, and dimeric IRF3 in cell lysates was separated from monomeric IRF3 by non-denaturing gel electrophoresis (Iwamura *et al.*, 2001). Western blot analysis was performed to detect IRF3, using a polyclonal rabbit antiserum (FL-425; Santa Cruz). IRF3, viral nucleoprotein (NP) and β-actin were also visualized in unfractionated cell lysates by standard Western blotting. (d) Western blot analysis of mouse embryo fibroblasts infected with the indicated viruses at an m.o.i. of 1. NS1, NP and β-actin were detected by using specific rabbit antisera (Solorzano *et al.*, 2005).

wild-type virus (Fig. 1c, lanes 2 and 6). As reported previously (Kochs et al., 2007a; Talon et al., 2000), a similar picture emerged when wild-type and NS1-deficient msPR8 were compared (Fig. 1c, lanes 8 and 9).

hvPR8 mutants with partial deletions of the NS1 gene were also generated that express N-terminal NS1 fragments of various lengths (aa 1–126, 1–99, 1–73). Proper expression of the expected NS1 fragments was confirmed by Western blot analysis of infected cells (Fig. 1d). IFN-β promoter activation by viruses with truncated NS1 was substantial, but remained at least 10-fold lower than activation observed by hvPR8-delNS1 (Fig. 1a). The viruses with truncated NS1 induced secretion of low levels of type I IFN into the supernatants of infected A549 cells (Fig. 1b). Accordingly, hvPR8(1–126) infection also triggered a low degree of IRF3 dimerization compared with hvPR8-delNS1 (Fig. 1c, lane 3). However, in cells infected with hvPR8(1–99) and hvPR8(1–73), IRF3 dimerization was not detectable (Fig. 1c, lanes 4 and 5).

It was of interest to determine the impact of the various NS1 truncations on virus virulence in mice carrying or lacking functional alleles of the IFN-induced Mx1 gene, which encodes a strong antiviral factor with specificity for influenza virus (Haller et al., 2007; Staeheli et al., 1986). As expected (Grimm et al., 2007), wild-type hvPR8 was highly virulent for $Mx1^{+/+}$ and $Mx1^{-/-}$ mice (Table 1). Remarkably, hvPR8-delNS1 showed a moderate degree of virulence in IFN-competent $Mx1^{-/-}$ mice. It killed 50 % of the infected animals if used at 10^5 f.f.u. (focus-forming units) per mouse. In contrast, hvPR8-delNS1 was non-pathogenic in $Mx1^{+/+}$ mice. It was also non-pathogenic in $Mx1^{+/+}$ $IFNAR1^{0/0}$ mice, which lack functional type I IFN receptors but carry functional Mx1 alleles (Mordstein et al., 2008). However, hvPR8-delNS1 was quite pathogenic for $Mx1^{+/+}$ $IFNAR1^{0/0}$ $IL28R\alpha^{0/0}$ mice, which lack functional receptors for both type I and type III IFN (Mordstein et al., 2008) (Table 1), confirming previous results suggesting that type III IFN confers partial protection against influenza A virus (Mordstein et al., 2008). Mutant hvPR8(1–126), expressing C-terminally truncated NS1, was surprisingly virulent in $Mx1^{-/-}$ mice. It was also highly virulent in $Mx1^{+/+}$ $IFNAR1^{0/0}$ $IL28R\alpha^{0/0}$ mice, but severely or moderately attenuated in $Mx1^{+/+}$ mice

carrying or lacking functional type I IFN receptors, respectively (Table 1).

To evaluate the capacity of the NS1-deficient hvPR8 mutants to induce IFN-β in vivo, we infected reporter mice in which the open reading frame of the IFN-β gene is replaced by the coding sequence of the firefly luciferase gene. Previous experiments showed that expression of luciferase in lung homogenates of such animals correlates with the induction of IFN-β (Lienenklaus et al., 2009). Infection with 5×10^4 f.f.u. hvPR8-delNS1 triggered a strong IFN response in the lungs of these reporter mice (Fig. 2a). This response was at least 20-fold stronger than the response elicited by wild-type hvPR8. Interestingly, under these conditions, msPR8-delNS1 showed no significant induction of the reporter gene; only if the virus dose was increased to 2×10^5 f.f.u. was reporter-gene expression observed with msPR8-delNS1 (Fig. 2a). Unlike msPR8-delNS1, hvPR8-delNS1 was able to replicate productively in lungs, even if the initial virus dose was only 5×10^4 f.f.u. (Fig. 2b). This result agrees with our finding that hvPR8-delNS1 is pathogenic in standard $Mx1^{-/-}$ mice (Table 1), whereas msPR8-delNS1 is not (Garcia-Sastre et al., 1998).

Strong expression of the IFN-β promoter-driven luciferase gene was also observed in reporter mice infected with hvPR8 mutants encoding C-terminally truncated NS1. In fact, reporter-gene expression by these viruses was about as high as that observed with hvPR8-delNS1 (Fig. 2a). At first glance, this result seemed to contradict our results with cultured cells, which showed clearly that hvPR8-delNS1 is superior (Fig. 1). To explain this discrepancy, one should take into account that the viruses with C-terminal truncations of NS1 grew much better in mouse lungs than hvPR8-delNS1. At 24 h post-infection, hvPR8 mutants with partial NS1 deletions had reached lung titres of 10^7–10^8 f.f.u., whereas hvPR8-delNS1 had reached lung titres of only 10^4–10^5 f.f.u. (Fig. 2b). Thus, even if the viruses with C-terminal NS1 truncations have a lower intrinsic IFN-inducing potential than hvPR8-delNS1, the higher replication capacity of the former viruses in the mouse lung eventually resulted in a comparably strong stimulation of the IFN system in vivo. The high replication phenotype of the NS1-truncated viruses might be explained by residual

Table 1. Virulence of hvPR8 viruses with NS1 deletions in mice

LD_{50} values (shown as f.f.u.) were determined by infecting groups ($n=4$) of wild-type and mutant mice (C57BL/6 genetic background) with various doses (10^1–10^6 f.f.u.) of the indicated viruses as described by Reed & Muench (1938). Animals were killed if severely ill or if weight loss approached 30 %. ND, Not done.

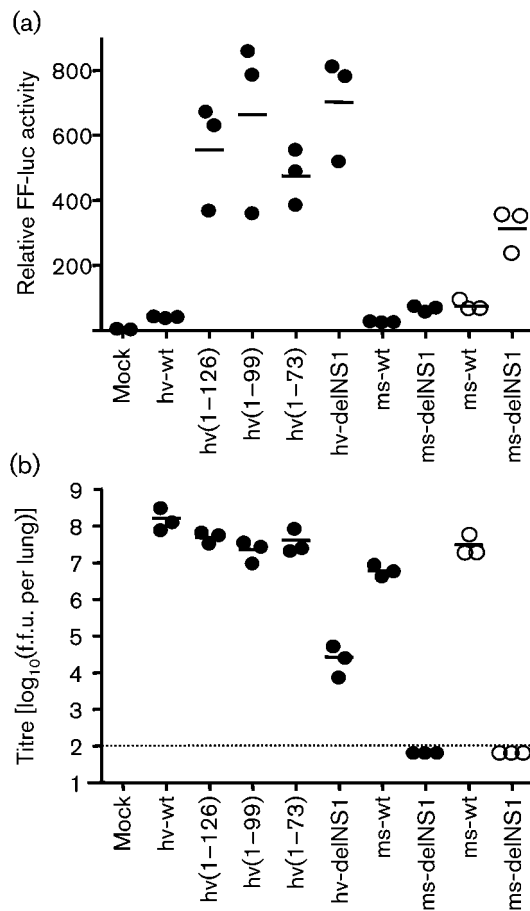| Virus strain	Mouse strains			
	$Mx1^{-/-}$	$Mx1^{+/+}$	$Mx1^{+/+}$ $IFNAR1^{0/0}$	$Mx1^{+/+}$ $IFNAR1^{0/0}$ $IL28R^{0/0}$
hvPR8-wt	<10	50	ND	ND
hvPR8(1–126)	<10	5×10^4	3×10^3	50
hvPR8-delNS1	10^5	$>2 \times 10^5$	$>2 \times 10^5$	10^3

Fig. 2. IFN-inducing activity correlates with virus replication of NS1 mutants in mice. (a) Virus-induced activation of the IFN-β promoter in transgenic reporter mice. Six- to eight-week-old mice expressing firefly luciferase (FF-luc) under the control of the IFN-β promoter (Lienenklaus *et al.*, 2009) were infected intranasally with either 5×10^4 f.f.u. (●) or 2×10^5 f.f.u. (○) of the indicated viruses. At 24 h post-infection, luciferase activity was determined in the lung homogenates of the reporter mice. (b) Virus replication in mouse lungs. Virus titres in lung homogenates of IFN-β reporter mice were determined as described previously (Grimm *et al.*, 2007). Mean values and data points of individual animals are shown. Representative data from one of two independent experiments are shown. The dotted line marks the detection limit.

anti-IFN activity of the N-terminal fragments (Kochs *et al.*, 2007b; Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005; Wang *et al.*, 2002). Alternatively, the N-terminal moiety of NS1 might facilitate virus replication per se, as NS1 has been shown to serve as cofactor of the viral polymerase complex (Falcon *et al.*, 2004; Kuo & Krug, 2009). It should be noted that other influenza virus strains with C-terminally truncated NS1 proteins also exhibit residual replication capacity in IFN-competent hosts (Egorov *et al.*, 1998; Kochs *et al.*, 2007b). However, unlike the hvPR8 mutants described here, these other viruses cannot grow to very high levels in mouse lungs in a very short time.

In conclusion, our study suggests that highly virulent viruses, such as hvPR8, tolerate mutations in NS1 surprisingly well. As such viruses replicate to high levels in infected tissues even if the IFN-antagonistic NS1 protein is crippled or absent, they trigger far more pronounced innate immune responses than their low-virulent counterparts. As hvPR8 is a mouse-adapted laboratory virus that may be handled in standard biosafety laboratories, hvPR8-derived NS1 mutants represent convenient novel tools for studying virus-induced expression of IFN genes *in vivo*.

Acknowledgements

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