

# Lethal H5N1 influenza viruses escape host anti-viral cytokine responses

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**The H5N1 influenza viruses transmitted to humans in 1997 were highly virulent, but the mechanism of their virulence in humans is largely unknown. Here we show that lethal H5N1 influenza viruses, unlike other human, avian and swine influenza viruses, are resistant to the antiviral effects of interferons and tumor necrosis factor  $\alpha$ . The nonstructural (NS) gene of H5N1 viruses is associated with this resistance. Pigs infected with recombinant human H1N1 influenza virus that carried the H5N1 NS gene experienced significantly greater and more prolonged viremia, fever and weight loss than did pigs infected with wild-type human H1N1 influenza virus. These effects required the presence of glutamic acid at position 92 of the NS1 molecule. These findings may explain the mechanism of the high virulence of H5N1 influenza viruses in humans.**

Avian (H5N1) influenza A viruses transmitted directly from chickens to humans in 1997 claimed the lives of six of the 18 people infected<sup>1-3</sup>. The cause of these viruses' virulence in humans is not known. Full post-mortem reports were available for two of the cases, and they described reactive hemophagocytic syndrome with elevated concentrations of the inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon (IFN- $\gamma$ )<sup>4</sup>. H5N1 influenza viruses were isolated only from the respiratory tracts of patients. Similarly, in a primate model of H5N1 influenza pathogenesis, virus was isolated from the respiratory tract but not from other organs, such as spleen, heart and brain<sup>5</sup>. In a mouse model, in contrast, H5N1 influenza virus was isolated from systemic organs, including the brain<sup>6,7</sup>. In humans infected with H5N1 influenza viruses, virus replication peaked 48 hours after infection, and little virus was shed after 6–8 days. The decline of viral replication corresponded closely to the level of circulating interferon<sup>8,9</sup>. Although both innate and induced immune responses are thought to have a role in virus clearance, IFNs and TNF- $\alpha$  are the first line of defense against the replication of influenza viruses<sup>10-12</sup>. We therefore investigated the inhibitory effect of these cytokines on lethal H5N1 influenza viruses directly transmitted from chickens to humans in 1997 (ref. 2).

## H5N1 influenza viruses are resistant to anti-viral cytokines

We treated St. Jude porcine lung (SJPL) epithelial cells with 200 ng/ml of recombinant swine IFN- $\alpha$ , IFN- $\gamma$  or TNF- $\alpha$  and then infected the cells with five H5N1 influenza viruses isolated in Hong Kong in 1997 (H5N1/97) at a multiplicity of infection (m.o.i.) of 0.0001. SJPL cells are the only available cell line that productively supports all subtypes of influenza virus<sup>13</sup>. As controls, we used human, swine, and avian influenza viruses. The replication of the H5N1/97 influenza viruses was not affected by pretreatment with IFN- $\alpha$ , IFN- $\gamma$  or TNF- $\alpha$  (Table 1). The titers of the H5N1/97 viruses were comparable in treated and untreated cells, whereas the replication of the control human, swine, and avian influenza viruses

was effectively blocked. Therefore, the highly pathogenic H5N1 influenza viruses have a novel mechanism that counteracts the antiviral activity of the interferons and TNF- $\alpha$ .

## Cytokine dose does not alter resistance of H5N1 viruses

To investigate the dose effect of the cytokines on the replication of H5N1/97 influenza viruses, we pretreated SJPL cells with 300 to 900 ng/ml of recombinant swine IFN- $\alpha$ , IFN- $\gamma$ , or TNF- $\alpha$  before infecting them with A/HK/156/97 (H5N1). The replication of H5N1/97 influenza viruses was not inhibited even at the highest concentration of cytokines (Fig. 1a). Virus titers were equivalent in pretreated and nonpretreated cells. Because treatment with the three cytokines yielded similar results, only the results obtained with IFN- $\gamma$  are shown.

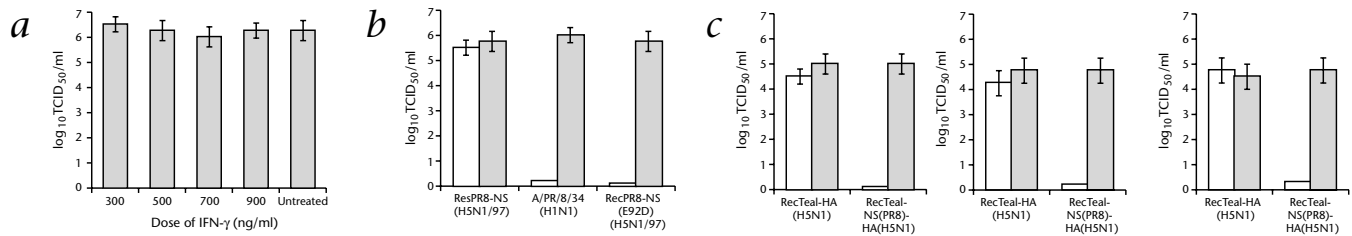
## NS gene of H5N1 viruses is required for cytokine resistance

Circulating influenza virus efficiently induces host expression of IFNs and TNF- $\alpha$  (refs, 8,14), which exert effective antiviral activity<sup>11,14</sup>. However, the NS1 protein encoded by influenza viruses is known to attenuate the host response mediated by interferons  $\alpha$  and  $\beta$  (refs. 15,16). To investigate the role of the NS gene in the resistance of H5N1/97 viruses to cytokines, we used reverse genetics<sup>17</sup> to create a recombinant A/PR/8/34 (H1N1) virus that contained the NS gene of A/HK/156/97 (H5N1). We designated the recombinant virus RecPR8-NS(H5N1/97). Pretreatment with 200 ng/ml of IFN- $\alpha$ , IFN- $\gamma$  or TNF- $\alpha$  did not inhibit the replication of the recombinant virus but effectively inhibited replication of the wild-type A/PR/8/34 virus (Fig. 1b). Because treatment with the three cytokines yielded similar results, only the results obtained with IFN- $\gamma$  are shown.

## Cytokine resistance requires glutamic acid at position 92

To determine which residue of NS1 is responsible for H5N1 viruses' resistance to the interferons and TNF- $\alpha$ , we compared the NS1 amino acid sequences of different influenza viruses.





**Fig. 1** Dose effect and roles of NS1 and HA in H5N1/97 viruses' resistance to cytokines. **a**, SJPL cells were pre-treated with the indicated concentrations of recombinant swine IFN- $\gamma$  before infection with A/HK/156/97 (H5N1) influenza virus. Virus titers were measured after 72 h. Values are the means of 3 independent experiments. **b**, SJPL cells pretreated ( $\square$ ) or not pretreated ( $\blacksquare$ ) with IFN- $\gamma$  were infected with recombinant A/PR/8/34 (H1N1) virus altered to encode the NS1 molecule of A/HK/156/97 (H5N1) virus [RecPR8-NS(H5N1/97)]; with the same recom-

binant virus encoding a Glu92Asp substitution in NS1 [RecPR8-NS(E92D)(H5N1/97)]; or with wild-type A/PR/8/34 (H1N1) virus. Virus titers were measured after 72 h. **c**, SJPL cells pretreated ( $\square$ ) or not pretreated ( $\blacksquare$ ) with IFN- $\gamma$  (left) TNF $\alpha$  (center) or IFN- $\alpha$  (right) were infected with recombinant A/Teal/HK/W312/97 (H6N1) virus containing the HA gene of A/Goose/HK/437-4/99 (H5N1) [RecTeal-HA(H5N1)] or with RecTeal-HA(H5N1) containing the NS gene of A/PR/8/34 (H1N1) [RecTeal-NS(PR8)-HA(H5N1)]. Virus titers were measured 72 h later.

Surprisingly, we found glutamic acid at position 92 of the H5N1/97 NS1 molecule. Other known human, equine, avian and swine influenza A viruses have aspartic acid at this position with the exception of H5N1/97 and H5N1/2001 influenza viruses and their progenitors<sup>18–20</sup>. We used site-directed mutagenesis to substitute aspartic acid (D) for glutamic acid (E) at this position of the A/HK/156/97 (H5N1) NS1 molecule. We then created a recombinant A/PR/8/34 (H1N1) virus that encoded the mutant NS1. The recombinant virus, designated RecPR8-NS(E92D)(H5N1/97), was susceptible to the antiviral activity of IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  (Fig. 1b). These results demonstrate that the presence of glutamic acid at position, 92 of the NS1 molecule is crucial to the H5N1/97 viruses' resistance to cytokines. Because treatment with the three cytokines yielded similar results, only the results obtained with INF- $\gamma$  are shown.

#### NS gene confers cytokine resistance to H5N1/2001 viruses

The H5N1/97 viruses disappeared from Hong Kong's live poultry markets after the wholesale slaughter of birds in 1997. In 2001, however, multi-reassortant H5N1 influenza viruses containing the hemagglutinin (HA) gene of H5N1/97 were isolated there. We investigated whether the H5N1/2001 influenza viruses are susceptible to the antiviral activity of IFNs and TNF- $\alpha$ . The replication of these viruses was not inhibited in SJPL cells pretreated with 200 ng/ml of INF- $\alpha$ , IFN- $\gamma$  or TNF- $\alpha$ . Virus titers were essentially identical in treated and untreated cells (Table 2). To investigate the role of the NS gene in these viruses' resistance to cytokines, we created recombinant virus by replacing the NS gene of A/PR/8/34 (H1N1) virus with the NS gene of A/Chicken/HK/FY150/01 (H5N1). This virus was designated RecPR8-NS(H5N1/2001). Its titers were comparable in SJPL cells pretreated with INF- $\alpha$ , INF- $\gamma$  or TNF- $\alpha$  and in untreated SJPL cells. Therefore, the NS gene is implicated in the H5N1/2001 viruses' resistance to the effects of cytokines. The NS genes of the H5N1/2001 viruses belong to allele A, as does the NS gene of H5N1/97. Interestingly, the NS1 of H5N1/2001 influenza viruses has a deletion of five amino acids (positions 79 to 83) and contains a glutamic acid at position 92. The NS1 amino acids of the H5N1/2001 influenza viruses had the following homology to NS1 of the A/HK/156/97 influenza viruses: 88.1% (A/CK/HK/FY150/01), 88.5% (A/PH/HK/FY155/01) and 87.2% (A/CK/HK/YU562/01).

#### HA is not implicated in H5N1 viruses' cytokine resistance

One of the unique features of the H5N1/97 influenza viruses is

their inclusion of multiple basic amino acids at the cleavage site of the HA protein<sup>21</sup>. To investigate the role of the HA protein in resistance to cytokine activity, we created two recombinant viruses by using A/Teal/HK/W312/97 (H6N1), whose genome is nearly identical to that of H5N1/97 (> 97% homology), with the exception of the HA gene<sup>18</sup>. We replaced the HA gene of this virus with that of the lethal A/Goose/HK/437-4/99 (H5N1) influenza virus, which is similar to that of H5N1/97 viruses. This recombinant was termed RecTeal-HA(H5N1). We then created a second recombinant virus by replacing the NS gene of RecTeal-HA(H5N1) with the NS gene of A/PR/8/34 (H1N1). This virus was designated RecTeal-NS(PR8)-HA(H5N1). After pretreatment with IFN- $\gamma$ , IFN- $\alpha$  or TNF- $\alpha$ , the titers of RecTeal-HA(H5N1) in treated cells were comparable to those in untreated cells (Fig. 1c). However, the replication of RecTeal-NS(PR8)-HA(H5N1) was almost completely blocked in pretreated SJPL cells. Therefore, the NS1 molecule, but not the HA molecule, is required for resistance to cytokines. The pathogenesis of influenza viruses in chickens and mice is reportedly associated with the presence of highly basic amino acids at the HA cleavage site<sup>22–24</sup>. The role of this feature in the pathogenesis of human infections remains unknown; however, the virus that caused the deadly 1918 'Spanish' flu pandemic did not have multiple basic amino acids at the HA cleavage site<sup>25</sup>.

The NS gene of H5N1/97 viruses is associated with virulence *in vivo*. To study the effect of the NS gene of H5N1/97 influenza virus on infection *in vivo*, we used A/PR/8/34 recombinant virus rather than H5N1 recombinant virus. This decision was based on a previous study in which infection with H5N1 viruses did not produce clear clinical signs in pigs<sup>21</sup>. We intranasally inoculated pigs with A/PR/8/34 (H1N1) or with recombinant A/PR/8/34 encoding the NS gene of A/HK/156/97 (H5N1) (RecPR8-NS(H5N1/97) and monitored virus shedding, body temperature and body weight. The shedding of both viruses reached a peak 2 days after inoculation, but the pattern of virus clearance differed (Fig. 2a). In pigs inoculated with A/PR/8/34 (H1N1), virus clearance began on day 3 after inoculation and was complete by day 5. In contrast, pigs inoculated with RecPR8-NS(H5N1/97) continued to shed virus at high titers for 6 days after inoculation and virus was cleared by day 10. The body temperature of the animals corresponded to the virus clearance cycle (Fig. 2b). Pigs inoculated with RecPR8-NS(H5N1/97) showed lethargy, shortness of breath, dehydration and severe loss of appetite. Pigs inoculated with A/PR/8/34(H1N1) and pigs inoculated with RecPR8-NS(H5N1/97) began to lose body weight 3 days after inoculation (Fig. 2c); however, those inocu-

**Table 1** Sensitivity of influenza viruses to interferons and TNF- $\alpha$ 

Viruses	Titer after pretreatment <sup>a</sup>			
	INF- $\alpha$	INF- $\gamma$	TNF- $\alpha$	No pretreatment
A/Hong Kong/156/97 (H5N1)	6.25	6.0	6.5	6.25
A/Hong Kong/483/97 (H5N1)	5.0	5.25	5.0	5.0
A/Hong Kong/486/97 (H5N1)	6.0	5.75	6.0	6.25
A/CK/Hong Kong/258/97 (H5N1)	7.0	7.25	7.25	8.0
A/Goose/Hong Kong/W374/97 (H5N1)	5.0	5.5	5.5	5.25
A/Hong Kong/1/68 (H3N2)	<	<	<	6.0
A/New Caledonia/20/99 (H1N1)	<	<	<	8.0
B/Lee/40	<	<	<	7.0
A/Swine/Ned/3/80 (H1N1)	<	<	<	7.25
A/Swine/NC/35922/98 (H3N2)	<	<	<	6.0
A/Mallard/Alberta/205/98 (H2N9)	<	<	<	5.5
A/Chicken/NY/13307-3/95 (H7N2)	<	<	<	4.25

<sup>a</sup>, SJPL cells were pretreated with 200 ng/ml of cytokine 24 h before infection with viruses (m.o.i., 0.0001). Virus titers ( $\log_{10}$ TCID<sub>50</sub>/ml) were measured 72 h after infection. Values are the means of 3 experiments. <:titer<1.

lated with RecPR8-NS(H5N1/97) had lost more than 40% of their original body weight by day 6, whereas the magnitude and duration of weight loss were relatively minor in pigs inoculated with PR/8/34(H1N1). These results implicate the NS gene of the H5N1/97 viruses in their pathogenicity *in vivo*.

### Virulence in pigs

Pigs inoculated with the recombinant virus RecPR8-NS (E92D)(H5N1/97), in which aspartic acid was substituted for glutamic acid at position 92 of NS1, had virus titers and clinical signs very similar to those of pigs inoculated with A/PR/8/34 (H1N1) (Fig. 2a–c). Therefore, the presence of glutamic acid at this position was crucial to the pathogenicity of the recombinant virus *in vivo*. Statistical analyses indicated that pigs inoculated with RecPR8-NS (H5N1/97) experienced significantly higher viral titers, body temperatures and loss of body weight than did pigs inoculated with A/PR/8/34 (H1N1) or RecPR8-NS(E92D)(H5N1/97) ( $P < 0.0001$ ). Position 92 of the H5N1/97 NS molecule is located near the known RNA-binding fragment (a 73-residue amino-terminal region). It remains to be determined how the presence of aspartic acid rather than glutamic acid at this position affects protein function<sup>26</sup>.

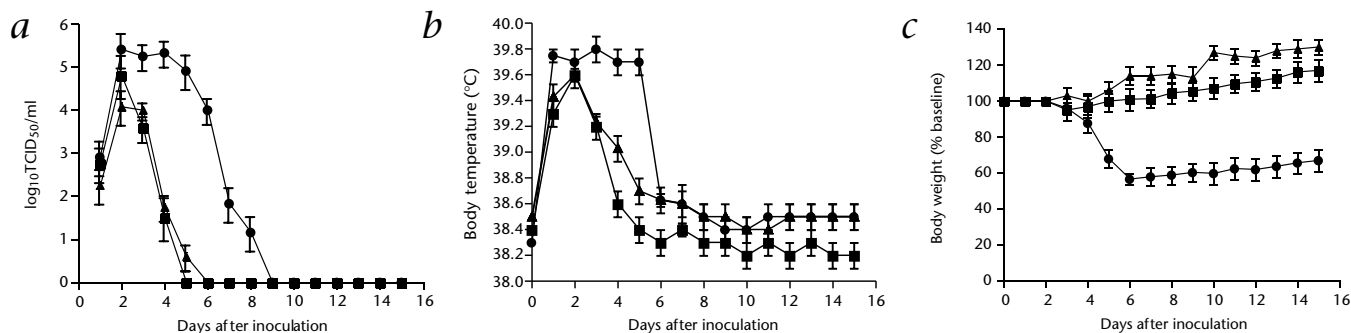
### Discussion

Our results show that H5N1 influenza viruses transmitted to humans in 1997 escape the antiviral activity exerted by the interferons and TNF- $\alpha$ . These cytokines are induced in the early phase of

infection and serve as the first line of antiviral defense<sup>27–29</sup>. In humans, the clearance of influenza virus is closely related to the quantity of circulating interferons and TNF- $\alpha$  (ref. 29). In the case of the H5N1/97 viruses, post-mortem reports of two human deaths suggested that virus replication in the respiratory tract caused high elevation of cytokines, including IFNs and TNF- $\alpha$ , and that hypercytokinemia and resultant reactive hemophagocytic syndrome were the chief cause of death<sup>4</sup>. It is possible that hypercytokinemia was caused or promoted in these cases by a mounting host cytokine response to influenza viruses that escaped the cytokines' antiviral effects and continued to replicate. Our previous study showed that the quantity of TNF- $\alpha$  expressed in lung ep-

ithelial cells depended on the dose of virus to which they were exposed<sup>13</sup>.

We showed that the NS gene of the H5N1/97 influenza viruses is implicated in their resistance to IFNs and TNF- $\alpha$ . Previous studies have shown that the NS1 gene of human influenza viruses functions as an antagonist of IFN- $\alpha/\beta$ . There are several mechanisms that may account for this antagonism. First, the influenza A NS1 protein inhibits the double-stranded RNA-activated protein kinase (PKR) *in vitro*, thereby inducing the phosphorylation of eukaryotic initiation factor 2 $\alpha$  and inhibiting protein translation<sup>30</sup>. Second, the influenza B NS1 protein blocks the conjugation of a ubiquitin-homolog IFN-stimulated gene of 15 kD (ISG15)<sup>16</sup>. And third, influenza A virus NS1 protein inhibits the activation of interferon regulatory factor 3 (IRF-3)<sup>31</sup>. Meanwhile, influenza A and B viruses efficiently induce expression of IFNs and TNF- $\alpha$  in hosts and pretreatment of cells with these cytokines blocks virus replication, despite the inhibition by NS1 protein of some immune events downstream of IFN- $\alpha/\beta$  expression<sup>10,11</sup>. Measles virus appears to use a related strategy to counteract the antiviral activity of IFN- $\alpha/\beta$ . Wild-type measles viruses, unlike attenuated measles viruses, interfere with induction of IFN in human peripheral blood mononuclear cells<sup>32</sup>. It remains to be discovered how the NS gene of lethal H5N1 influenza virus allows the virus to escape the effects of IFNs and TNF- $\alpha$ . We propose that the NS gene of these viruses is resistant to degradation by unknown anti-viral proteins and that it binds and inactivates these proteins in cells. Anti-viral



**Fig. 2** Role of the NS gene in the resistance of H5N1 virus to cytokines *in vivo*. **a–c**, Groups of 5-wk-old pigs were intranasally inoculated with A/PR/8/34 (H1N1) influenza virus (■), with recombinant A/PR/8/34 (H1N1) virus containing the H5N1/97 NS gene [RecPR8-NS(H5N1/97)] (●) or with RecPR8-NS(H5N1/97) virus encoding a Glu92Asp substitui-

tion in NS1 [RecPR8-NS(E92D)(H5N1/97)] (▲). Nasal swab specimens were obtained and body weight and temperature were recorded daily for 15 d. Panels show changes in virus titers (a), body temperature (b) and body weight (c). Each point represents the mean result obtained from 3 pigs.

proteins can easily degrade the NS genes of other circulating influenza viruses, thereby inactivating the remainder of the viral genes.

The mechanism that allows lethal H5N1 influenza viruses to survive in cells treated with interferon or TNF- $\alpha$  remains to be determined. However, this mechanism appears to depend on the substitution of glutamic acid for aspartic acid at position 92 of the NS1 molecule. In a similar study by Basler *et al.*<sup>33</sup>, a recombinant human A/WSN/33 (H1N1) influenza virus containing the NS gene of the 1918 'Spanish' flu virus, A/Brevig Mission/1/18 (H1N1), was less pathogenic in mice than the wild-type A/WSN/33 (H1N1) virus. Amino acid comparison showed that A/Brevig Mission/1/18 (H1N1) had aspartic acid at position 92 of NS1, as do other influenza viruses. It would be worthwhile to investigate whether other isolates of the 1918 influenza virus have glutamic acid at that position. Because protein activity may depend on conformation, we cannot rule out the possibility that other amino acids in the NS1 of H5N1/97 are involved in its resistance to antiviral cytokines.

The hemagglutinin of the lethal H5N1/97 influenza viruses is not involved in their resistance to IFNs and TNF- $\alpha$ , although it possesses a unique feature (multiple basic amino acids at its cleavage site)<sup>19</sup> that is associated with pathogenicity in chickens<sup>22,23</sup>. In a mouse model, the high cleavability of H5N1 influenza virus HA was reported to be essential for lethal infection, but a mutation at position 627 in the PB2 protein influenced the rate of mortality<sup>24</sup>. The role of HA cleavability in the pathogenesis of human influenza virus infections remains uncertain. The tissue tropism of H5N1 viruses appears to differ in humans and mice. H5N1/97 viruses were isolated only from the respiratory tracts of humans<sup>4</sup> but were isolated from multiple organs of mice, including the brain<sup>6,7</sup>. Further, the 1918 'Spanish' flu, which killed 20–40 million humans worldwide, did not have multiple basic amino acids at the HA cleavage site<sup>25</sup>.

All of the H5N1/97 isolates we tested escaped the antiviral activity of IFNs and TNF- $\alpha$ . Previous studies showed that A/HK/483/97 (H5N1), which was isolated from a fatal human case, was highly pathogenic in inbred mice, causing a systemic infection that included the brain; however, A/HK/486/97 (H5N1), which was isolated from a mild case of human disease, had low pathogenicity in inbred mice, causing only respiratory infection<sup>6,7,24</sup>. We showed that both isolates escape the antiviral effects of IFNs and TNF- $\alpha$ . The pathogenicity of viruses may be influenced by many factors: the virus gene constellation, genetic background, host immune status and infectious dose. It is possible that the A/HK/486/97 (H5N1) virus, which was isolated from a mild human case, could cause fatal disease in other humans. The findings of a study in ferrets confirm this possibility. Outbred ferrets infected experimentally with A/HK/483/97 or with A/HK/486/97 showed comparable clinical signs: fever, weight loss, nasal discharge and lethargy; and both viruses were isolated from the nasal turbinates, lungs and extrapulmonary organs, including the brain<sup>34</sup>. The human immune status may be an important factor in disease outcome. A study of memory CD8<sup>+</sup> and CD4<sup>+</sup> cytotoxic T lymphocytes (CTL) in humans showed that some CTL lines are cross-reactive against both A/HK/156/97 (H5N1) and A/HK/483/97 (H5N1)<sup>35</sup>.

The pathogenicity of influenza viruses is a polygenic trait. If our *in vivo* studies had used the H6N1 and H9N2 strains, which carry the E92D mutation of the NS gene in the context of other

**Table 2** Sensitivity of H5N1 (2001) influenza viruses to interferons and TNF- $\alpha$

Viruses	Cytokines <sup>a</sup>			Untreated
	INF- $\alpha$	INF- $\gamma$	TNF- $\alpha$	
A/Chicken/Hong Kong/FY150/01 (H5N1)	5.25	5.5	5.0	5.25
A/Pheasant/Hong Kong/FY155/01 (H5N1)	5.0	4.5	5.0	5.0
A/Chicken/Hong Kong/YU562/01 (H5N1)	5.25	5.0	5.5	5.0
RecPr8-NS (H5N1/2001)	4.75	4.5	5.25	4.75
PR8	<	<	<	5.25

<sup>a</sup>, SJPL cells were pretreated with 200 ng/ml of cytokine 24 h before infection with viruses (m.o.i., 0.0001). Virus titers (log<sub>10</sub>TCID<sub>50</sub>/ml) were measured 72 h after infection. Values are the means of 3 experiments. <: titer < 1.

genes, the clinical outcome might have been different. However, the ability to escape the first line of the host's immune defense may be an important determinant of the pathogenicity of influenza viruses in humans, in whom interferon plays an important role in reducing the viral burden<sup>10</sup>.

An appropriate small animal model would greatly facilitate studies of influenza virus pathogenesis. However, our experiments indicate that studies in pigs and ferrets are more informative than those in mice, because most inbred mice are deficient in one or more interferon pathways<sup>36</sup>. The ferret is the currently accepted model for determining influenza pathogenesis. However, the miniature pig is recognized as an intermediate influenza virus host and is susceptible to all influenza subtypes. Reagents are increasingly available for immunologic analysis of these animals and we anticipate their increased use in studies of pathogenesis.

In conclusion, the highly pathogenic H5N1 influenza viruses transmitted to humans in 1997 have a novel mechanism that circumvents the powerful protective effects of IFNs and TNF- $\alpha$ . It is crucial to prevent further transmission of these influenza viruses to humans by continuous surveillance and the design of an effective vaccine.

## Methods

**Cell culture and viruses.** St. Jude porcine lung (SJPL) epithelial cells were cultured as described<sup>27</sup> and used for antiviral activity assays. Madin–Darby canine kidney (MDCK) cells were cultured in MEM supplemented with 10% FBS. 293T human embryonic kidney cells were cultured in Opti-MEM I (Life Technologies, Gaithersburg, Maryland) containing 5% FBS. Influenza viruses used in this study were propagated in 10-d-old chicken eggs. The lethal H5N1 influenza viruses used in this study were handled only in a BL3<sup>+</sup> biocontainment facility by trained staff.

**Generation of recombinant viruses.** To make recombinant viruses by reverse genetics, we cloned cDNA of the NS gene of A/Hong Kong/156/97 (H5N1) or A/Chicken/Hong Kong/YU562/01 (H5N1) influenza virus into the plasmid pHW2000 (ref. 17). Briefly, viral RNA was isolated by using the RNeasy kit (Qiagen, Valencia, California) according to manufacturer's instructions. The RNA was reverse transcribed and amplified by PCR (SuperScript Preamplication System, Life Technologies) by using NS-specific primers (forward: TATTCGTCTCAGGGAGCAAAGCAGGTTG; reverse: ATATCGTCTCG-TATTAGTAGAAACAAGGTTGTTT). PCR products were purified with the QIAquick PCR purification kit (Qiagen), digested with *BsmBI* at 55 °C and ligated into the pHW2000 expression vector. A/PR/8/34 (H1N1) and A/Teal/HK/W312/97 (H6N1) viruses were cloned as described<sup>17</sup>.

**Recovery of recombinant viruses.** Viruses created by transfection of plasmids were recovered as described<sup>17</sup>. Briefly, MDCK and 293T cells (1 × 10<sup>6</sup> cells per well of a 6-well plate) were co-cultured for 24 h before transfection. One  $\mu$ g of each plasmid and 2  $\mu$ l of TransIT-LT-1 (Panvera, Madison, Wisconsin) per  $\mu$ g of DNA were mixed with Opti-MEM I (total volume, 200  $\mu$ l) in a 1.5-ml Eppendorf tube and incubated for 45 min at room temperature. The mixture was then diluted with 800  $\mu$ l of Opti-MEM I and added to the cells. The trans-

fectected cells were incubated at 37 °C for 30 h and 1 ml of Opti-MEM I containing 1 µg/ml of TPCK-trypsin was added. The rescued viruses were allowed to replicate in MDCK cells before being used in the antiviral assay.

**Antiviral activity assay.** The antiviral activity of the cytokines was assayed as described<sup>13</sup>. Briefly, SJPL cells were cultured in DMEM with 10% FBS in 6-well tissue culture plates. Cells were pretreated for 24 h with the indicated quantities of recombinant swine TNF-α (R&D Systems, Minneapolis, Minnesota), swine INF-γ (R&D Systems) and swine INF-α (Biosource International, Camarillo, California) and then infected with influenza viruses at an m.o.i. of 0.0001. The low m.o.i. allowed accurate detection of infectious viral titers in SJPL cells (in which influenza viruses grow readily) 72 h after inoculation. The infected cells were incubated for 72 h with DMEM containing 0.3% BSA and 1 µg/ml trypsin treated with L-(1-tosylamido-2-phenyl)ethylchloromethylketone (TPCK). The virus titer of the supernatant medium (log<sub>10</sub> median tissue-culture infective dose per milliliter; log<sub>10</sub>TCID<sub>50</sub>/ml) was measured in SJPL cells grown in 96-well plates. TPCK-trypsin was not added for the antiviral assays of H5N1 influenza viruses.

**Site-directed mutation.** We used PCR with overlapping primers to substitute aspartic acid for glutamic acid at position 92 of A/HK/156/97 influenza virus NS1. We created recombinant viruses that incorporated this gene with those of A/PR/8/34 (H1N1) virus as described<sup>15</sup>. The recombinant virus was designated RecPR8-NS(E92D)(H5N1/97). The two internal primers were 5'-ACCTAACTGACATGACTCTTGAGGAAATGTCAAGG-3' and 5'-CCTTGACATTTCTCAAGAGTCATGTGAGTTAGGT-3'.

**Inoculation of pigs with viruses.** Groups of three 5-wk-old Yucatan miniature pigs (Charles River Laboratories, Windham, Maine) were inoculated intranasally with 0.6 ml of a 1 × 10<sup>6</sup> TCID<sub>50</sub>/ml suspension of A/PR/8/34 (H1N1), RecPR8-NS(H5N1/97) or RecPR8-NS(E92D)(H5N1/97). The pigs' nostrils were swabbed daily to quantify virus shedding, and their body weight and temperature were measured daily. Infection experiments were performed in a BL3+ containment facility. The pigs were housed in laminar flow cabinets and were handled by a single person who wore protective clothing and a fitted HEPA filter mask.

**Statistical analysis.** Viral titers, body temperature and body weight were compared by analysis of variance. Two-way layouts with replicates were used for all pair-wise comparisons. A P-value <0.05 was prospectively defined as indicating a statistically significant relationship.

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#### Competing interests statement

The authors declare that they have no competing financial interests.

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