Shortening the unstructured, interdomain region of the non-structural protein NS1 of an avian H1N1 influenza virus increases its replication and pathogenicity in chickens

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Currently circulating H5N1 influenza viruses have undergone a complex evolution since the appearance of their progenitor A/Goose/Guangdong/1/96 in 1996. After the eradication of the H5N1 viruses that emerged in Hong Kong in 1997 (HK/97 viruses), new genotypes of H5N1 viruses emerged in the same region in 2000 that were more pathogenic for both chickens and mice than HK/97 viruses. These, as well as virtually all highly pathogenic H5N1 viruses since 2000, harbour a deletion of aa 80–84 in the unstructured region of the non-structural (NS) protein NS1 linking its RNA-binding domain to its effector domain. NS segments harbouring this mutation have since been found in non-H5N1 viruses and we asked whether this 5 aa deletion could have a general effect not limited to the NS1 of H5N1 viruses. We genetically engineered this deletion in the NS segment of a duck-origin avian H1N1 virus, and compared the in vivo and in vitro properties of the WT and NSdel8084 viruses. In experimentally infected chickens, the NSdel8084 virus showed both an increased replication potential and an increased pathogenicity. This in vivo phenotype was correlated with a higher replicative efficiency in vitro, both in embryonated eggs and in a chicken lung epithelial cell line. Our data demonstrated that the increased replicative potential conferred by this small deletion was a general feature not restricted to NS1 from H5N1 viruses and suggested that viruses acquiring this mutation may be selected positively in the future.

INTRODUCTION

The genome of influenza A viruses (IAVs) consists of eight ssRNAs of negative polarity, which code collectively for up to 15 proteins (Jagger et al., 2012; Muramoto et al., 2013; Palese & Shaw, 2007; Wise et al., 2009). IAVs continue to pose a threat to human and animal health, notably the H5N1 highly pathogenic avian influenza viruses that have become endemic in Asia and other parts of the world. Following their appearance, circulating H5N1 viruses have undergone
a complex evolution (Duan et al., 2008; Nguyen et al., 2012; Vijaykrishna et al., 2008), which involved several reassortment events with other avian influenza viruses. Among the eight genetic segments of currently circulating H5N1 viruses, only segments 4 and 6, encoding the haemagglutinin (HA) and neuraminidase (NA), respectively, can be traced back to the progenitor Goose/Guangdong/1/96 (Gs/GD) virus. The other segments have undergone several exchanges and most of them continue to be exchanged through reassortment (Duan et al., 2008). Segment 8, which encodes the non-structural (NS) protein NS1 and the nuclear export protein (NEP, formerly named NS2), exists as two distinct alleles, A and B, with allele B being found exclusively in some viruses of avian origin. The progenitor Gs/GD virus had an allele B-NS, which was replaced rapidly by an allele A-NS in the Hong Kong/156/97 (HK/97)-like viruses.

After the successful eradication of HK/97 viruses, multiple new genotypes of H5N1 (i.e. A–E and V–Z) emerged in the Hong Kong region in the early 2000s (Guan et al., 2002a, b; Li et al., 2004), that were characterized by their high pathogenicity for chickens, and also for mice without prior adaptation, and their unusual capacity to spread to the brain (Guan et al., 2002a). These viruses had notably acquired a mutation in the NS segment, resulting in deletion of aa 80–84 in the unstructured region linking the RNA-binding domain (RBD) of NS1 to its effector domain (ED) (Table S1 and data available in the online Supplementary Material). Segment 8 carrying this deletion has since become largely predominant among H5N1 viruses (Duan et al., 2008) and has also propagated to some non-H5N1 viruses (Munir et al., 2013) (Fig. 1, Table S2).

**Fig. 1.** NS1 types in Asian H5N1 viruses, 1996–2012. Fully sequenced NS1-ORFs from H5N1 viruses isolated in Asia were searched in the Influenza Virus Resource database and grouped in three classes: allele B NS1 (●), allele A with no deletion (■) and allele A with the aa 80–84 deletion (del8084) (▲). Sequences harbouring a premature stop codon or suppression of the usual stop codon were not taken into account. The thin dashed line represents the total number of sequences. Numbers of sequences are shown on a logarithmic scale.

NS1, a ~230 aa protein, is absent from the virion but highly expressed in the infected cell, where it exhibits several pro-viral activities (Hale et al., 2008b; Marc, 2012): (i) it binds to the 30 kDa subunit of cleavage-and-polyadenylation specificity factor (CPSF30) and to several components of the mRNA export machinery (Nemeroff et al., 1998; Satterly et al., 2007; Shapira et al., 2009), thereby preventing the maturation and nucleocytoplasmic export of cellular mRNAs; (ii) it regulates the splicing of the viral mRNA encoding M1 (Robb & Fodor, 2012) and enhances translation of the viral mRNAs (Márion et al., 1997); and (iii) it prevents the triggering of the IFN system and blocks IFN-mediated antiviral activities through its interaction with several proteins acting in the IFN response (Fernandez-Sesma et al., 2006; Gack et al., 2009). Its N-terminal RBD, which is a dimer of aa 1–73, interacts with many different RNA species and notably with specific motifs in the viral (+)strand RNAs (Marc et al., 2013), whilst its C-terminal ED is involved in most of its interactions with proteins. NS1 is necessary for normal growth of the virus and genetically engineered viruses with invalidated NS1, or devoid of NS1, are impaired severely in their replication (Cauthen et al., 2007; Donelan et al., 2003; Egorov et al., 1998; García-Sastre et al., 1998; Kochs et al., 2007).

Whether and how the two domains of NS1 interact with each other is unknown. However, because each of its two domains can dimerize independently (Bornholdt & Prasad, 2006; Hale et al., 2008a; Nemeroff et al., 1995), NS1 can form either single dimeric units or long oligomeric structures of alternating RBD and ED dimers (Bornholdt & Prasad, 2006, 2008). Moreover, the existence of several dimerization
modes of the ED may allow NS1 to adopt distinct quaternary states (Aramini et al., 2011; Kerry et al., 2011). Switching between these states may be relevant to NS1 activities in the infected cell, and may to some extent depend on the length and flexibility of the unstructured region linking the two domains (Bornholdt & Prasad, 2008; Kerry et al., 2011). Therefore, shortening the linker region through deletion of aa 80–84 may alter the activities of NS1 (Bornholdt & Prasad, 2008) and thus the replication properties of viruses harbouring this NS1 variant (hereafter named del8084).

The establishment of this internal NS1 deletion in circulating H5N1 viruses, and its presence in some other avian and canine isolates, not only indicates that it is not detrimental, but rather suggests that this modification could enhance the viral fitness, possibly by enhancing the pro-viral activities of NS1. Two previous studies have addressed the putative pro-viral effects of the aa 80–84 deletion in the context of an H5N1-origin NS1. Li et al. (2010) examined the impact of the aa 80–84 deletion on various biological activities of a transiently expressed H5N1-NS1 in human cell culture systems, but failed to detect a distinct effect other than a slightly decreased NF-kB activation following TNF-α treatment in H5N1-NS1del8084-transfected cells versus H5N1-NS1-transfected cells. Long et al. (2008), using a recombinant A/WSN/33(H1N1) strain, found that introduction of the 5 aa deletion in the linker region of NS1 enhanced pathogenicity in both chickens and mice, but the authors did not provide further details about the viral phenotype (e.g. with respect to in vitro and/or in vivo growth kinetics, pathological lesions, and the cytokine expression pattern).

To assess whether the 5 aa deletion could have a general effect not restricted to the NS1 of H5N1 viruses, we genetically engineered this deletion in the NS segment of a duck-origin avian H1N1 virus, and compared the properties of the WT and NSdel8084 viruses in a comprehensive (in vivo and in vitro) experimental approach using recombinant avian influenza viruses and NS1 expression vectors. In addition, we analysed the cytokine responses in chickens infected by the two different viruses and assessed the IFN-α/β antagonistic activities of transiently expressed WT-NS1 or del8084-NS1.

**RESULTS**

A search through the Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) revealed that from 2001 onwards, virtually all H5N1 viruses in Asia harboured the NS segment with the 15 nt deletion, hereafter named del8084-NS1, which first appeared in 2000 (Figs 1 and 2, and supplementary data). Importantly, whilst the few H5N1 viruses harbouring full-length NS1 (hereafter named fl-NS1) were isolated only in avian species, those harbouring del8084-NS1 were isolated in a large panel of species, including mammals (Li et al., 2004). Moreover, the H5N1-origin segment 8 encoding the del8084-NS1 has now also been found in some viruses from non-H5N1 subtypes, including two canine influenza viruses (Table S2). This, along with the unusual pathogenicity of the new genotypes of H5N1 viruses, is what prompted us to investigate to what extent the 5 aa deletion in NS1 may impact the viral phenotype in a non-H5N1 avian influenza virus.

**Viral replication in inoculated chickens**

We genetically engineered two variants of the duck H1N1 isolate A/Mallard/Marquenterre/Z237/83 (Munier et al., 2010), which expresses a fl-NS1 (Fig. 2). The recombinant WT virus (MZ) harbours a segment 8 identical to that of the parental isolate, whilst the virus named NSdel8084 harbours a modified segment 8 encoding a del8084-NS1. We then compared the properties of the two viruses in specific-pathogen-free histocompatible B13/B13 White Leghorn chickens (4 weeks old) that were inoculated intra-tracheally with 2 × 10^7 EID_{50} (50 % egg infective dose) of WT or NSdel8084 viruses. At days 0 (6 h), 1, 2, 3, 4 and 7...
post-infection (p.i.), tissue samples were taken for viral RNA (vRNA) quantification from three to five euthanized birds. vRNA was extracted and the levels of M-vRNAs were determined by quantitative reverse transcription (qRT)-PCR. All mock-inoculated chickens were negative for the presence of vRNAs (data not shown). At days 0 and 1, viral loads in the lungs were similar between the two groups (viral loads at day 0 likely corresponded to the inoculum) (Fig. 3).

As was observed previously with the MZ virus (Munier et al., 2010), the highest viral loads were recorded at 2 and 3 days p.i. At these two time points, viral loads in the lungs were significantly higher in NSdel8084-inoculated chickens (Fig. 3a, two-tailed P=0.015 for the grouped day 2+3 values), whereas viral loads at later time points (days 4 and 7 p.i.) were similar between the two viruses. Thus, replication of the MZ WT virus in the lungs seemed to be delayed compared with that of NSdel8084. In the upper respiratory tract, vRNA could be quantified in the oropharyngeal swabs from days 1 to 4 p.i. From days 2 to 4, only two of 12 of the oropharyngeal swabs were positive in WT-inoculated animals compared with eight of 12 in the NSdel8084-inoculated chickens (Fig. 3c).

Histological lesions in inoculated chickens

Lesions of the lungs were observed only in virus-inoculated chickens, both at the macroscopic level during necropsy (Fig. 3e, f), and through histological analysis of fixed lung samples that were collected at days 1, 2, 3, 4 and 7 p.i. (Fig. 3g, j). Peribronchiolar interstitial pneumonia (Fig. 3g, j) was more frequent and more pronounced with the NSdel8084 virus than with the WT virus. Foci of infiltrating mononuclear inflammatory cells scattered in the lung parenchyma around airways were indicative of interstitial pneumonia. The lesions were more extensive in NSdel8084- as compared with WT-inoculated chickens for all time points of the study (Fig. 3g). These signs of interstitial pneumonia also persisted longer in NSdel8084-inoculated chickens compared with WT-inoculated birds in which lesions were only minimal at day 7 p.i. In addition, airway lesions were found as early as day 1 p.i. in the lower respiratory tract, and were associated with intraluminal presence of a fibrinous exudate and some degenerating heterophils, typical of exudative bronchitis and parabronchitis. This exudate obstructed airways in severe cases. Focal extensive necrosis of the bronchial respiratory epithelium was noticed from day 1 p.i. in all NSdel8084-inoculated chickens, whereas only some WT-inoculated chickens presented scant amounts of intraluminal epithelial debris from day 3 p.i. No lesions were observed in the other examined tissues.

Induction of cytokine mRNAs in infected chickens

Total RNAs were prepared from the lungs of chickens euthanized and necropsied at days 0, 1, 2, 3 and 4 p.i. The levels of cytokine mRNAs were measured using qRT-PCR and were normalized with respect to the geometric mean of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), G10 and ubiquitin mRNA levels. As shown in Fig. 4, only IFN-α and IL-6 showed clear patterns of overexpression in the lungs (Fig. 4a, c). We did not observe an unambiguous overexpression of the Mx mRNA in infected birds (Fig. 4b). For days 2–4 p.i., we plotted the expression levels of IL-6 against those of IFN-α in order to highlight the correlation between these two values (Fig. 4d). We observed that the two groups of virus-infected chickens formed two distinct clusters, reflecting the fact that NSdel8084-infected chickens had the highest values of both IL-6 and IFN-α between days 2 and 4. Between these two time points, IFN-α mRNA levels in NSdel8084-inoculated chickens were 100–1000 times higher than those in WT-inoculated chickens (two-tailed P<0.029 for each time point between days 2 and 4). We also observed that at days 2 and 3 p.i., the elevated expression levels of IFN-α mRNA in NSdel8084-inoculated birds were correlated strongly with the viral load in the lungs. At day 4, both IL-6 and IFN-α remained overexpressed in the lungs of NSdel8084-infected chickens (100–1000 times higher in this group when compared with the WT-infected group) in spite of decreasing viral loads, probably reflecting a persistent inflammatory response after the peak of viral replication.

Faster replication kinetics of NSdel8084 in cultured cells and in ovo

We next asked whether the increased replication efficiency of NSdel8084 that we observed in vivo could be observed in vitro. We set up a multicycle growth assay in CLEC213 cells, a chicken lung epithelial cell line described previously (Esnault et al., 2011). In this system, NSdel8084 grew to titres that were 3- to 10-fold higher than those obtained with the WT virus (Fig. 5a). The growths of the two viruses were also compared in embryonated eggs, revealing that the NSdel8084 virus grew to HA titres that were significantly higher than those of the WT virus (Fig. 5b).

No difference between the two NS1 variants in the modulation of the IFN type I response

We then sought to determine whether the two variants of NS1 might regulate the antiviral IFN response differentially.
As the antiviral response in virus-infected cells could be biased by the differing replication efficiencies of the WT and NSdel8084 viruses, we instead opted to measure the liposome–poly(I:C) complex [L-poly(I:C)]-induced antiviral response in cells which transiently expressed either variant of the NS1 protein. L-poly(I:C) is an efficient MDA5 agonist and it was shown previously that, even in the absence of RIG-I from the chicken genome, RIG-I-like receptor signalling is the principal IFN type I-inducing pathway triggered by IAV in chicken cells (Liniger et al., 2012). Both NS1 variants were expressed transiently in CLEC-213 cells by transfection of recombinant pCI plasmids encoding either WT-NS1 or del8084-NS1. The two variants of NS1 were expressed to similar levels, as verified by a Western blot assay using a rabbit anti-NS1 polyclonal serum (data not shown). Control cells were transfected with the empty vector pCI. Transfected cells were then stimulated by a L-poly(I:C) treatment and the IFN type I response was measured by a dual-luciferase assay assessing the transcriptional activity of the Mx promoter. When compared with the corresponding untreated cells, the L-poly(I:C) treatment induced a 31-fold overexpression of the Mx promoter in empty vector-transfected cells (Fig. 6) as compared with 15- and 17-fold overexpression in WT-NS1- or del8084-NS1-expressing cells.

**DISCUSSION**

In order to investigate whether the deletion of aa 80–84 in the interdomain region of NS1 could confer new properties to avian influenza viruses, irrespective of their subtype or origin, we genetically engineered two variants of a duck H1N1 virus, with the sole difference between the two variants consisting in the presence or absence of the 15 nt deletion in segment 8 RNA. Importantly, this deletion does not alter the spliced mRNA encoding NEP (NS2). We compared the pathogenicity of the two viruses in a chicken model of IAV infection. Despite the naturally low pathogenicity of the MZ virus for chickens (Munier et al., 2010), we observed marked differences between the two viruses. The NSdel8084 virus replicated to higher levels, both in the lung and in the upper respiratory tract of experimentally infected chickens, and spread to internal tissues (kidneys) much more efficiently than its WT counterpart, likely as a result of blood transport. However, unlike what we observed in a similar chicken infection model with an unrelated low-pathogenicity avian H7N1 virus (Soubies et al., 2013), in the present study we found no virus in the brain, which indicates that this mutation by itself does not confer a neurotropic phenotype, at least in the chicken model and for this virus (H1N1 MZ). The more efficient replication of the NSdel8084 virus in the upper respiratory tract probably favours its airborne dissemination, which may account for the evolutionary success of H5N1 viruses harbouring such a modified segment 8. Higher replication in the respiratory tract also led to more pronounced lung parenchyma lesions and elicited more extensive inflammatory infiltrates in that tissue, notably at days 2 and 3 p.i., which correlates with the observed overexpression of pro-inflammatory cytokine IFN-β and IL-6 mRNAs in the lungs of the infected chickens. This overexpression was correlated with the level of viral replication and as a consequence it was much higher in the lungs of the NSdel8084-inoculated chickens, notably at days 2, 3 and 4 p.i.

Our results are in good agreement with previous observations showing that recombinant viruses harbouring this short deletion in the NS segment had increased pathogenicity and increased replication potential, in both chickens and mice (Long et al., 2008). Taking into account the known properties of NS1, the difference between the two NS variants may depend on: (i) their IFN type I antagonistic activities; (ii) their interaction with viral or cellular RNAs (maturational, nucleocytoplasmic export, translation); or (iii) their putative function in regulating the synthesis of vRNAs (Falcon et al., 2004; Min et al., 2007; Wang et al., 2010). The higher replication efficiency of NSdel8084 in chickens correlated with the same phenotype in vitro, both in a multicycle growth assay in the chicken lung epithelial cell line CLEC213 (Ensaut et al., 2011) and in embryonated eggs. This observation prompted us to examine whether the transcription/replication activity of MZ-derived ribonucleoproteins transiently reconstituted in avian cells (as described in Labadie et al., 2007) was regulated differentially upon co-expression of the WT-NS1 or del8084-NS1 protein. However, no significant effect of any of the two variants of NS1 was observed under these experimental conditions (data not shown). With respect to the antiviral IFN type I response, we cannot attribute the differences in IFN-α and IL-6 mRNA expression levels to distinct anti-IFN properties of the two NS1 variants, as these differences appear to be correlated primarily with the distinct replication efficiencies of the two viruses in the chicken model of infection. Furthermore, if the NSdel8084 variant of NS1 would exhibit more efficient anti-IFN activities, one would expect to see a lower IFN response in the lungs of the NSdel8084-infected chickens; on the contrary, we observed a much stronger expression of IFN-α in these birds, at least at the mRNA level (Fig. 4a). In addition, transient expression of WT-NS1 and del8084-NS1 in L-poly(I:C)-stimulated CLEC213 cells revealed similar IFN type I antagonistic activities for the two NS1 variants, as was assessed by a luciferase-based Mx reporter assay. Similar observations were made by Li et al. (2010) using H5N1-NS1 expression vectors harbouring the 5 aa NS1 deletion and a luciferase-based IFN-β or IFN-sensitive response element reporter system. Therefore, the del8084 variant of NS1 appears to provide a growth advantage to IAVs that is not linked to the well-established IFN-inhibitory activities of NS1. This advantage may result from the combination of subtle effects either on the replication machinery or on the metabolism of viral and cellular RNAs. At present, no precise molecular mechanism can be proposed to explain how the shortening of NS1’s linker region can impact its activities, although previous studies have shown that both the sequence and the length of
Fig. 3. Viral loads and lesions in the tissues of the inoculated chickens. (a–c) Tissue viral loads were expressed as the log_{10} of the copy number of M-vRNA mg^{-1} or per swab (WT, Δ; NSdel8084, ▲). (d) Viral loads in the kidney were plotted against viral loads in the lung, from days 2 to 4. (e, f) Representative macroscopic pathological findings in lungs from a WT virus-inoculated.
Shortened NS1 linker augments influenza virus fitness

this linker region can impact the viral phenotype (Li et al., 2011; Long et al., 2008) and the quaternary structure of NS1 (Carrillo et al., 2014). Our minimal hypothesis to explain the observed effect of this deletion is that a shortened linker region would alter the quaternary structure and constrain the relative mobility of the two domains of NS1. This in turn could alter the equilibrium between distinct quaternary states of NS1 that may be relevant to its functions (Aramini et al., 2011; Kerry et al., 2011). One of the possible quaternary states consists in the oligomerization of NS1 on its ligand RNA, which has been demonstrated in several independent studies (Aramini et al., 2011; Bornholdt & Prasad, 2008; chicken (e), showing a large inflammatory region in the lower left region of the lung) and from a control chicken (f) at day 4 p.i. (g) Histological lesions were scored as described in Methods. (h–j) Lesions observed in lungs collected from chickens at day 1 after inoculation with WT-MZ (i) or NSdel8084-MZ virus (j), in comparison with a lung from a control mock-inoculated bird (h). Parabronchial (p) and antrum (a) lumens are empty. (i) Large foci of interstitial peribronchiolar pneumonia are observed. Inflammatory cells (o) partially filled the terminal airways including parabronchiae and infiltrated the surrounding parenchyma. (j) Large coalescing foci of interstitial peribronchiolar pneumonia are observed. Terminal airways are occluded by large amounts of fibrin. The surrounding parenchyma is severely infiltrated by inflammatory cells. Haematoxylin–eosin–safron stain. Bar, 150 μm.

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Fig. 4. Cytokine mRNA expression in the lungs. Total RNA was extracted from the lungs of WT-inoculated chickens (△), NSdel8084-inoculated chickens (▲) and mock-inoculated chickens (●) at days 0–4 p.i. (a–c) The levels of the indicated cytokine mRNAs at the indicated time points were determined using qRT-PCR. The results are expressed as mRNA copy numbers (y-axis, left scale) normalized with respect to 10^7 copies of the geometric mean of three reference gene cDNA copy numbers (GAPDH, G10 and ubiquitin), as measured in the same sample. Values from the mock-inoculated chickens were grouped. The median value for each experimental group is indicated by a horizontal bar. (d) For days 2–4 (grouped), IL-6 mRNA levels were plotted against IFN-α mRNA levels in the two groups (WT, green; NSdel8084, red), with individual symbol size reflecting the lung viral load in the corresponding lungs (dots, <10^4; circles, 10^4–10^6; large circles with central dot, >10^6 copies mg⁻¹).
Marc et al., 2013). Oligomerization of NS1 could play a role in the nucleocytoplasmic export of viral mRNAs and in the regulation of their splicing. The shortened interdomain linker of NS1 could favour its oligomerization, or result in more compact oligomers that could enhance the nucleocytoplasmic export of viral mRNAs and therefore increase the virus fitness. In addition to its impact on oligomerization, the shortened linker region could also alter the equilibrium between the ‘helix-open’ and ‘helix-closed’ quaternary states of NS1 that have been proposed to be involved in distinct activities of NS1 (Kerry et al., 2011). Clearly, there is a need for a better understanding of the structure-based mechanical aspects of NS1 functions at the molecular level and in particular about the relative mobility of its two domains. It is yet unknown whether, as has been observed in other RNA-binding, multidomain proteins (Kowalinski et al., 2011), there is a conformational change of NS1 associated with RNA binding that could possibly induce new activities in NS1 and be affected by a deletion in the interdomain linker. The existence of such a mechanism would be consistent with our data, which reveal that the interdomain length is a general determinant of IAV replicative capacity, not restricted to H5N1 viruses. Thus, viruses acquiring this deletion through reassortment with H5N1 viruses may exhibit an increased fitness and may be selected positively in the future. The fact that this modified segment 8, of H5N1 origin, has been found in several distinct subtypes of non-H5N1 viruses since 2002, on several occasions and in distantly related geographical locations (Table S2), argues in favour of this possible evolution.

**METHODS**

**Viruses and cells.** The H1N1 IAV A/Mallard/Marquenterre/ Z237/83 (MZ) was isolated by the National Influenza Centre (Northern France) at the Institut Pasteur in Paris (France). After introduction of the 15 nt deletion into the NS reverse-genetics plasmid using the QuikChange II kit (Stratagene), the two recombinant IAVs (WT-MZ and NSdel8084-MZ), both derived from the MZ virus described above, were rescued by reverse genetics as described previously (Munier et al., 2010). Stocks of the two viruses that were compared throughout this study were prepared by two rounds of amplification in embryonated chicken eggs (10 days old) at an m.o.i. of 2 p.f.u. per egg, followed by two further rounds of amplification on MDCK cells. MDCK and chicken lung epithelial CLEC213 cells (Esnault et al., 2011) were grown in Eagle’s minimal essential medium and Dulbecco’s modified Eagle’s medium (DMEM-F12), respectively, supplemented with 7.5% FCS.

![Fig. 5. Viral growth on avian cultured cells and in embryonated eggs. (a) Multicycle growth in avian CLEC213 cells that were infected at an m.o.i. of 0.001. Virus in the supernatant was titrated on an MDCK layer (mean ± SEM, n=3 independent replicates). (b) Both viruses were titrated in embryonated eggs (10 days old). HA titres were recorded in each of the HA-positive eggs.](image1)

![Fig. 6. Modulation of Mx promoter expression by transient expression of NS1. CLEC213 cells were transfected with expression vectors encoding either variant of NS1 or with empty vector. At 24 h after transfection, they were either stimulated by a L-poly(I : C) treatment or left untreated. At 24 h after L-poly(I : C) treatment, cells were lysed and the Mx-driven expression of firefly luciferase was measured and normalized to Renilla luciferase activity. Bars correspond to the Mx-driven overexpression (fold change) in L-poly(I : C)-treated cells relative to the corresponding untreated cells (mean ± SEM; n=3 wells per condition). Data are representative of two independent experiments with similar results.](image2)
**Multicycle growth kinetics.** Subconfluent monolayers of chicken lung epithelial cells (CLEC213) were prepared in 75 cm² flasks (Essault et al., 2011). They were infected with either virus at an m.o.i. of 0.001 p.f.u. per cell. Following 1 h of adsorption at 37 °C, the cells were further incubated in serum-free DMEM containing 0.4 μg L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (Worthington). Supernatant samples taken at 12, 24, 48, 60 and 72 h p.i. were titrated subsequently by plaque assays on MDCK cells as described previously (Matrosovich et al., 2006).

**Plasmids and luciferase reporter assays.** Eukaryotic expression vectors encoding WT-NS1 (pCIwt-NS1) or del8084-NS1 (pClde8084-NS1) were constructed by subcloning the respective coding sequences between the XhoI and NotI sites of the pCI plasmid (Promega). In order to prevent production of spliced mRNAs, splice-donor and splice-acceptor sites were both invalidated by point mutations (Talon et al., 2000). Using Lipofectamine 2000 transfection reagent (Life Technologies), subconfluent monolayers of CLEC213 cells in 24-well plates were co-transfected with a mixture of three different plasmids: (i) 0.1 μg per well of either pCI (empty vector control), pCIwt-NS1 or pClde8084-NS1 plasmid, together with (ii) 0.1 μg per well of pGL3-chMx-Luc encoding the firefly luciferase under the control of the chicken Mx promoter (Liniger et al., 2012) (kindly provided by Dr Nicolas Ruggli, IVI, Mittelhäusern, Switzerland) and (iii) 1 ng per well of pRl-CMV (Promega) encoding the Renilla luciferase as a transfection control. At 24 h post-transfection, cells were either treated with 1 μg ml⁻¹ of the RIG-I/MDA-5 agonist LyoVec-poly(1:C) [L-poly(1:C); InvivoGen] or left untreated (n=3 wells for each condition). At 24 h after L-poly(1:C) treatment, cells were lysed, and activation of the chicken Mx promoter was revealed by using the dual-luciferase reporter assay system (Promega) and a GloMax-Multi microplate luminometer (Promega).

**Chicken experiments.** All animals were kept and treated in strict compliance with the Good Animal Practice rules as defined by the relevant national and local animal welfare authorities, and all animal experimental interventions were approved by the local ethics committee (approval July 2010, Comité d’Ethique pour l’Expérimentation Animale, Région Val de Loire).

SPF histocompatible B13/B13 White Leghorn chickens (4 weeks old) were housed in biosafety level 3 cabinets under negative pressure with HEPA-filtered air. Briefly, three groups of 23 (WT-MZ), SPF histocompatible B13/B13 White Leghorn chickens (4 weeks old) were housed in biosafety level 3 cabinets under negative pressure with HEPA-filtered air. Briefly, three groups of 23 (WT-MZ), 23 (NSde8084-MZ) and 10 (mock) birds were inoculated with 2 × 10⁸ EID₅₀ of the two different viruses in a 0.2 ml volume via the intratracheal route, whilst in the mock group the virus suspension was replaced by PBS. Birds were carefully monitored daily during the course of the trial (1 week). Three to five birds at days 1, 2, 3 and 4 p.i. and three birds at day 7 p.i. for each virus-inoculated group were euthanized and necropsied, along with two additional mock-inoculated birds at each of the indicated times. Swabs and tissue samples from lung, kidney, spleen and caecum were collected from each animal, and either frozen at −80 °C until use for further downstream analyses (vRNA quantification and cytokine mRNA quantification) or fixed in 10% neutral buffered formalin for subsequent histopathological evaluation.

**Quantification of vRNAs.** Tissue samples (50 mg) were transferred in 1 ml PBS and dissociated mechanically using a tissue grinder (Retsch). The QiaAmp Viral RNA Mini Kit (Qiagen) was used to prepare vRNAs from 140 μl samples of tissue homogenates, according to the manufacturer’s recommendations. For the quantification of M-vRNAs by real-time RT-PCR, the Superscript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) and a Chromo 4 thermocycler (Bio-Rad) were used, according to the manufacturer’s recommendations. The sequences of the primer pairs targeting the M-segment as well as the qRT-PCR conditions were described previously (Munier et al., 2010).

**Quantification of cellular cytokine and chemokines mRNAs.** Total RNA was extracted from lung samples after dissociation with TRIzol (Sigma) according to the manufacturer’s recommendations and treated with RNase-free DNase I (Invitrogen). RT was performed on 1 μg RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s recommendations. Amplification of the cDNA by qPCR using a Chromo 4 thermocycler was performed in triplicate with 2 μl twofold-diluted cDNA samples, 7.5 μl 2 × iQ SYBR Green Supermix (Bio-Rad), 4 μl ultrapure water (Invitrogen) and 0.75 μl each specific primer (10 μM). Expression levels for mRNAs of chicken IFN-α, Mx, IL-6 and three reference genes GAPDH, G10 and ubiquitin cDNA copy numbers, as measured in the same sample.

**Histopathological evaluation.** Formalin-fixed tissues were embedded in paraffin wax, and 6 μm thick sections were cut and routinely stained with the haematoxylin–eosin–safron procedure. Histological observation was then performed and each lesion recorded. For lung tissue, semiquantitative assessment of broncho-interstitial lesions was performed and the corresponding individual score attributed as follows: 0, no lesion; 1, focal presence of bronchial lesions associated with moderate influx of mononuclear cells in the surrounding parenchyma; 2, multifocal presence of bronchial lesions with marked peripheral influx of inflammatory cells occupying 10–30 % of the lung parenchyma; 3, multifocal bronchial lesions with severe infiltration of >30 % of the parenchyma by inflammatory cells.

**Statistical analyses.** vRNA copy numbers as determined by qRT-PCR in RNA extracts from tissue homogenates were analysed as follows: (i) values below the detection threshold, i.e. 200 copies, were arbitrarily replaced by 1; and (ii) copy numbers were converted to their log₁₀ values. When indicated, the two sets of viruses were compared using a Mann–Whitney test with a two-tailed P value, as for histological lesions. Cytokine mRNA copy numbers determined on lung samples from mock-, WT- or NSde8084-inoculated chickens were first compared using a Kruskal–Wallis test in order to detect any virus-induced changes. The values obtained for the virus-inoculated chickens were further compared using a Mann–Whitney test. Analyses were performed using GraphPad Prism 5.0.

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