Generation and characterization of a new panel of broadly reactive anti-NS1 mAbs for detection of influenza A virus

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Influenza A virus (IAV) non-structural protein 1 (NS1) has multiple functions, is essential for virus replication and may be a good target for IAV diagnosis. To generate broadly cross-reactive NS1-specific mAbs, mice were immunized with A/Hong Kong/1/1968 (H3N2) 6xHis-tagged NS1 and hybridomas were screened with glutathione S-transferase-conjugated NS1 of A/Puerto Rico/8/1934 (H1N1). mAbs were isotyped and numerous IgG-type clones were characterized further. Most clones specifically recognized NS1 from various H1N1 and H3N2 IAV types by both immunoblot and immunofluorescence microscopy in mouse M1, canine Madin–Darby canine kidney and human A549 cells. mAb epitopes were mapped by overlapping peptides and selective reactivity to the newly described viral NS3 protein. These mAbs detected NS1 in both the cytoplasm and nucleus by immunostaining, and some detected NS1 as early as 5 h post-infection, suggesting their potential diagnostic use for tracking productive IAV replication and characterizing NS1 structure and function. It was also demonstrated that the newly identified NS3 protein is localized in the cytoplasm to high levels.

INTRODUCTION

Influenza A virus (IAV) is a negative-sense ssRNA virus with a segmented genome (reviewed by Palese & Shaw, 2007; Wright et al., 2007). IAV belongs to the family Orthomyxoviridae and is subtyped based on its surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). IAV circulates in humans and causes seasonal influenza infection with mild to severe illness but can also be life-threatening for adults, infants and immunodeficient people (Tan et al., 2010). IAV also undergoes substantial antigenic variation, due to the low fidelity of RNA transcription and the segmented nature of the viral genome (Lindstrom et al., 1998). In addition, 17 HA and 10 NA types have been identified so far (Tong et al., 2012; reviewed by Palese & Shaw, 2007). Genetic mixing of various genes, including the HA and NA genes, has led to the emergence of new strains that contribute to pandemics. IAV contains eight segments of RNA encoding ten to 15 viral proteins (reviewed by Jagger et al., 2012; Palese & Shaw, 2007; Wright et al., 2007).

The viral non-structural protein NS1 is encoded by viral RNA segment 8 and is essential for virus replication in normal cells (reviewed by Hale et al., 2008). NS1 is a relatively small multifunctional protein of 202–237 aa (Dundon, 2012). Based on phylogenetic analysis, IAV NS1 proteins can be divided into two major groups: allele A, which contains the NS1 proteins of human, swine, avian and equine IAVs, and allele B, which contains only NS1 proteins of avian influenza viruses (Ludwig et al., 1991; Treanor et al., 1989). All human seasonal influenza viruses contain allele A NS1. Most IAV antigenic changes are
driven by selective pressures on the surface antigens HA and NA, and there is no correlation between the surface subtypes and the antigenic properties of NS1 (Brown et al., 1983). NS1 interacts with both RNA (Hatada & Fukuda, 1992; Hatada et al., 1992) and numerous host proteins, including RIG-I (Guo et al., 2007; Mibayashi et al., 2007; Opitz et al., 2007), TRIM25 (Gack et al., 2009), CPSF30 (Kochs et al., 2007a; Nemeroff et al., 1998), PABPN1 (Chen et al., 1999) and NXXF1/TAP (Satterly et al., 2007). NS1 appears to modulate viral gene splicing (Fortes et al., 1994; Garaigorta & Ortín, 2007; Robb et al., 2011) and is also involved in restraining host immune responses by inhibiting the production of interferon and interferon-induced proteins, which play important roles in antiviral activities (Bergmann et al., 2000; Egorov et al., 1998; García-Sastre et al., 1998; Jia et al., 2010; Kochs et al., 2007b; reviewed by Hale et al., 2008). We recently identified and characterized a panel of adaptive mutations that enhance interferon antagonism of the A/Hong Kong/1/1968 (H3N2) NS1 protein (Forbes et al., 2012), one of which resulted in the generation of an alternatively spliced variant protein product, NS3, that possesses an in-frame deletion of aa 126–168 (Selman et al., 2012). Subcellular distribution of NS1 protein in infected cells depends on the virus strain, cell type and procedure of fixation. Although synthesized in the cytoplasm, large amounts of this non-structural viral protein are found in the cytoplasm and nucleus (Greenspan et al., 1988; Hale et al., 2008; Li et al., 1998; Newby et al., 2007) and in nuclear ND10 structures (Sato et al., 2003). NS1 has recently been demonstrated by computational analyses to contain 21 functional and 15 structural ‘sequence features’ (Noronha et al., 2012; Squires et al., 2012), and several key amino acid residues play important roles in determining viral and host protein intracellular distribution (see, for example, Ayllon et al., 2012).

As NS1 is the only IAV non-structural protein identified in all strains and is expressed in abundance after infection (Krug & Etkind, 1973), it may be a good target for detection and diagnosis of productive IAV infection. Previous studies have generated mAbs against highly pathogenic H5N1 NS1 that were useful for viral detection and titration purposes (Rai et al., 2010; Tan et al., 2010). In addition, NS1-targeted mAbs have been generated previously against A/WSN/1933 (H1N1) to analyse the antigenic variation in NS1 of a wide range of influenza viruses of different origins (Brown et al., 1983). However, the most prevalent currently circulating human IAVs are of the H1N1 and H3N2 subtypes, and, as indicated above, there are a disproportionately large number of sequence features in the small, critically important viral NS1 protein. Thus, we sought to generate additional broadly cross-reactive, yet highly NS1-specific, mAbs that might be useful for characterizing this important viral protein from a wide range of viral subtypes and to define additional sequence features.

RESULTS AND DISCUSSION

Anti-NS1 mAb production and isotyping

IAVs undergo considerable genetic and antigenic variation among their constituent proteins. Phylogenetic analyses of various IAV genes have indicated that some, such as polymerase protein genes, are relatively highly conserved, whereas the major surface antigenic determinant proteins, HA and NA, are more variable (Palese & Shaw, 2007; Wright et al., 2007). Assessment of variation among six laboratory strains that we routinely work with demonstrated these relative patterns among the encoded proteins (Fig. 1). We observed that there was 96–99% sequence identity between the PA proteins of three of our H1N1 strains, PR8, B59 and NCal (see Table 1 for viral strain abbreviations), 97–99% sequence identity between our H3N2 PA proteins in HK1, NY55 and B10, and 95–96% sequence identity between any two H1N1 and H3N2 PA proteins (Fig. 1a). Comparisons of the HA proteins of this panel of viruses showed 79–97% sequence identity between any two H1N1 strains and 86–98% identity between any two H3N2 strains but only 38–41% sequence identity between any H1N1 and H3N2 HA proteins (Fig. 1a).

Similar comparative analysis of the NS1 protein showed intermediate values. There was >90% identity between any two H1N1 members and >92% identity between any two H3N2 group members but ~83–85% identity when comparing any H1N1 NS1 protein with any H3N2 NS1 protein (Fig. 1a). Among this small group of prototype virus strains, the NS1 proteins of PR8 and HK1 were the most divergent (data not shown). For this reason, we adopted the strategy of selecting for broadly cross-reactive, yet NS1-specific, antibodies by immunizing with NS1 derived from the HK1 strain and screening hybridomas with NS1 derived from the PR8 strain.

After immunizing female BALB/c mice with 6 × His-tagged HK1 protein, spleen cells were collected and fused with myeloma cells and several hundred potential monoclonal hybridomas were screened for binding to GST–PR8-NS1 by ELISA. An initial Western blot screen identified several dozen positive hybridomas, which were then cloned by sequential limiting dilution twice. We continued analysis of the 13 strongest-reacting clones. Immunoglobulin isotyping indicated that one clone was IgM and 12 clones were IgG. Subtyping of the IgG types indicated that five were IgG1 and 12 IgG2a, four were IgG2b, two were IgG1 and one was IgG3. Among these 12 IgG mAbs, 10 were able to detect recombinant GST–NS1 by Western blotting. Nine of these mAbs were successfully purified by protein G affinity chromatography and used for subsequent studies (Table 2).

Anti-NS1 antibodies are specific for NS1 from multiple virus strains

Each of the IAV strains, except HK1, which was employed in later assays, were initially used to infect Madin–Darby...
**Fig. 1.** Phylogenetic comparisons of various influenza virus proteins. (a) Percentage identities between selected H1N1 clones (open bars), selected H3N2 clones (shaded bars) and H1N1 and H3N2 clones (filled bars) of each of the influenza virus proteins. PA, PB1 and PB2, polymerase proteins; NP, nucleoprotein; M1 and M2, matrix proteins; NEP, nuclear export protein. (b) Sequence alignment of selected NS1 proteins. The sequence region in HK1 denoted by a black line above the sequences (aa 125–167) is missing in a recently described NS3 protein identified in a mouse-adapted strain (Selman et al., 2012). The strains are identified in Table 1. The five boxes indicate epitopes mapped to particular mAbs (indicated below the boxes) as determined by subsequent epitope mapping. (c) Western blot with anti-NS1 mAb 3F5 for different strains of IAV. MDCK cells were infected with the indicated IAV strains (or mock infected) and cells were lysed in lysis buffer containing 0.5 % NP-40. The proteins were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted with mAb 3F5. (d) Slot-blot analysis of the anti-NS1 mAbs. MDCK cells were infected (or mock infected) with the indicated IAV strains for 24 h. Cell lysates were prepared and the cytosolic fractions were bound to PVDF membranes. Identical concentrations of the indicated mAbs were tested against identical concentrations of lysate. Bound antibodies were detected with goat anti-mouse secondary antibody and enhanced chemiluminescence.
canine kidney (MDCK) cells, and cell lysates were collected at 24 h post-infection (p.i.), resolved by SDS-PAGE and immunoprobed with mAb 3F5. The 3F5 antibody reacted cleanly with a single protein, resulting in a strong positive single band at an apparent molecular mass of ~26 kDa, which corresponds to the molecular mass of NS1. Similar reactivity was found for all five tested IAV-infected samples, and was not present in the mock-infected sample (Fig. 1c). The small variation in apparent molecular masses probably reflects the sequence differences between these strains. A much fainter band was occasionally observed at a higher molecular mass of ~42 kDa, which may represent modified forms or oligomers of NS1. When mAb 3F5 was subsequently used to immunoprecipitate proteins from PR8-infected MDCK cell lysates, a single 26 kDa protein (in addition to the IgG chains) was observed following SDS-PAGE, and mass spectrometry identified this protein as PR8 NS1 (data not shown).

As analysis of the first antibody indicated that it bound NS1 with little if any background, we then used a slot-blot apparatus to rapidly screen each of our nine antibodies against mock-infected lysates, as well as lysates infected with each of five IAV strains. All nine mAbs were affinity purified on protein G columns and used in all remaining experiments. MDCK cell lysates (25 μg) infected with each IAV strain were bound to a PVDF membrane and probed with identical concentrations (1 μg) of each purified mAb. Eight of the purified IgG mAbs (13D8, 5D6, 10C7, 8C7, 5F4, 4E10, 3F5 and 7D11) detected the denatured form of NS1 of all five test IAV strains, whilst the ninth mAb, 5B10, bound denatured NS1 of only PR8, NY55 and NCal (Fig. 1d). The mAbs also demonstrated different affinities. 5D6 recognized the NS1 proteins of most strains to almost the same extent, consistent with few alterations within the amino acid sequence (Fig. 1b), as determined by subsequent epitope mapping. Similarly, 10C7, although it clearly had a higher affinity as demonstrated by stronger reactivity, also reacted nearly equally with most tested clones, with reactivity to NCal NS1 being the exception. However, a number of mAb clones demonstrated strain-specific reactivity. For example, 4E10 reacted most strongly with PR8 NS1 and less strongly with NS1 derived from other tested clones, all of which have sequence variation in the region subsequently determined to contain the epitope.

We then performed immunofluorescent microscopy to test the capacity of the mAbs to bind to the native form of NS1 and to observe subcellular NS1 distribution. This assessment was initiated with mAb 3F5. This mAb reacted with

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*Reactivities are indicated as: ++++, strong; +, medium; +/−, weak; and −, none. Values represent composite scores, based on reactivities against multiple test viruses, as shown in Figs 1–3.

†Epitopes were determined by Pepscan Therapeutics using linear overlapping peptide arrays as described elsewhere (Slootstra et al., 1996). Values correspond to amino acid positions within NS1.
all tested IAV strains, although 3F5 appeared to react more strongly with B59- and NCal-infected MDCK cells and more weakly with B10-infected MDCK cells (Fig. 2). NS1 was found in both the cytoplasm and nucleus at the 24 h time point used for this initial assay. Similar analyses of other IAV strains with other mAbs showed variability in their capacities to recognize NS1 proteins from different IAV strains (Fig. 3). For example, 5F4 and 13D8 reacted with all tested strains, whereas 5D6, 8C7 and 10C7 reacted very weakly, if at all, with B10, and all five tested mAbs detected PR8 and B59. Thus, several of these new mAbs recognized both denatured and native forms of several NS1 proteins.

**PR8 NS1 is detected as early as 5 h p.i. by Western blotting**

Time-course studies of MDCK cell infections indicated that the earliest time point at which PR8 NS1 could be detected by Western blotting was 5 h p.i. (Fig. 4a). Detection of PR8 NS1 by immunofluorescence microscopy was less sensitive, and NS1 generally was not detectable before 6–8 h p.i. (Fig. 4b). In addition, inspection of these images indicated that the subcellular distribution of NS1 changed over time. NS1 was detectable initially in subnuclear structures where it remained for several hours. Cytoplasmic staining became more apparent after 12 h p.i., and subnuclear staining was also apparent at this time. Cytoplasmic staining became more even and diffuse with longer incubation times, and the subnuclear staining appeared to diminish or disappear (Fig. 4b).

**Epitope mapping of the new NS1 mAbs**

We recently described a panel of adaptive mutations in human IAV that were selected through several serial passages in mice (Forbes *et al.*, 2012; Selman *et al.*, 2012). These mouse-adapted variants of HK1 possessed...
several mutations in the NS1 protein, including a D125G amino acid substitution (GAT→GGT). The D125G mutation also induced an alternatively spliced transcript that resulted in deletion of an internal motif in the NS1 protein corresponding to aa 125–167. We called this novel splice variant protein NS3 (Selman et al., 2012). We tested our NS1 mAbs for their reactivity to both wild-type HK1 NS1 protein and the NS1 D125G mutant and NS3 proteins, grown in human A549 cells (data not shown) and mouse M1 cells, to determine whether any of the mAbs recognized an epitope within the deleted region. Most mAbs that detected HK1 NS1 also reacted with the NS3 protein. However, three mAbs, (3F5, 5F4 and 13D8) reacted with the HK1 NS1 but not NS3 (Fig. 5a), indicating these mAbs react with an epitope(s) located between aa 125 and 167. In addition, a fourth mAb (5B10) reacted with NS3 but only at low levels. To define more precisely the epitopes recognized by each mAb, aliquots of each antibody were submitted to Pepscan Therapeutics for overlapping peptide library analysis. Each mAb reacted with a defined set of peptides and allowed mapping of the epitope of each mAb (Fig. 5b). Five different epitopes were identified. Most mAbs, such as 3F5 and 5B10, reacted with a limited number of linear peptides and generated single well-defined peaks. The 3F5 mAb reacted strongly with peptides only between NS1 aa 160 and 169, and therefore its epitope could be assigned to the sequence 138FDRLETILL147, overlapping with the previously described nuclear export signal sequence feature (Noronha et al., 2012; Squires et al., 2012) (Table 2). Similarly, 29DAPFLDR35, located within the RNA-binding domain, was the epitope recognized by 7D11. 29DAPFLDR35 was highly conserved amongst the tested IAV strains (Fig. 1b) and therefore this mAb reacted well with the NS1 of numerous tested IAV strains (Fig. 1d). Several mAbs appeared to react with several linear peptides located in different regions of the NS1 protein. For example, 8C7 reacted with several peptides near aa 160 and 210 (Fig. 5b), possibly due to cross-reactivity to these similar epitope regions possessing SPLPSLPGH and PPLTPKQK, respectively. To determine more precisely the respective contributions of each of these regions, we performed competitive ELISA, by treating plate-bound GST–PR8-NS1 with an excess of each of the various mAbs and then testing the capacity of biotinylated mAb 8C7 to react. Biotinylated 8C7 bound efficiently to NS1 pre-treated with no antibody, an irrelevant antibody that recognizes β1 integrin or mAb 7D11, which recognizes a distant epitope (Fig. 5c). Biotinylated 8C7 binding was reduced to background levels by 5F4 and by non-biotinylated 8C7, by ~80% by 3F5 and by ~30% by 4E10. These results, combined with the capacity of 8C7 to recognize NS3 (Fig. 5a), suggested that 8C7 does recognize both epitopes. All nine of the mAbs epitopes could be

**Fig. 3.** Several NS1 mAbs detect various NS1 proteins. MDCK cells were infected (or mock infected) with the indicated IAV strains at an m.o.i. of 1, fixed at 24 h p.i. and probed with the indicated mAbs. Bar, 20 μm.
mapped on the primary NS1 amino acid sequence map (Table 2) and eight were placed within the deduced three-dimensional NS1 atomic structure (Fig. 5d–f). The two mAbs that recognized 211RPPLT215 (4E10 and 8C7) bound near the disordered carboxyl terminus, which lacks a defined structure.

**Discrimination of NS1 from NS3 in infected cells**

The previous Western blot screening analyses of mAb binding to HK NS1 and NS3 proteins demonstrated that 3F5, 5F4 and 13D8 bound the spliced-out intronic region of NS1 that is absent from the NS3 protein. Thus, these mAbs can be used specifically to detect NS1 in the presence of NS3. We performed confocal microscopy with a rabbit polyclonal antibody to detect both NS1 and NS3, and mAb 3F5 to detect only NS1 protein, in mouse M1 cells infected with HK1 wild-type virus that only expressed NS1 or with the NS1 M124I/D125G mutant expressing both NS1 and NS3 (Fig. 6). 3F5 staining of NS1 protein in HK1 wild-type NS1- and the M124I/D125G-infected M1 cells at 16 h p.i. was primarily nuclear, with a low level of cytoplasmic

**Fig. 4.** Kinetics of NS1 detection. (a) NS1 mAb can detect NS1 at 5 h p.i. in Western blots. MDCK cells were infected with PR8 at an m.o.i. of 5, cell lysates were obtained at the indicated time points (h p.i.) and proteins were resolved by SDS-PAGE and transferred to PVDF membrane. NS1 was probed with mAb 3F5 and β-actin was used as the loading control. Primary antibodies were detected with goat anti-mouse antibody and enhanced chemiluminescence. M, Mock infected. (b) NS1 can be detected by 8 h p.i. by immunofluorescent microscopy. A time course of NS1 production was carried out over a span of 18 h. MDCK cells were infected with PR8 at an m.o.i. of 5, harvested at the indicated time points (h p.i.) and prepared for immunofluorescence microscopy. NS1 was stained with primary mAb 8C7 and secondary Alexa Fluor 546-conjugated antibody (red). Note the presence of punctate formations in the nuclei of MDCK cells at 12 h p.i. F-actin was stained with phalloidin (green) and the nuclei were stained with DAPI (blue). The column on the right shows enlargements of the indicated boxed regions in the merged images. Bar, 20 μm (far-right column); 50 μm (other images).
staining detected with the M124/D125G mutant associated with higher NS1 expression (Forbes et al., 2012). Polyclonal antibody staining of parallel samples also showed nuclear staining of the HK1 wild-type-infected cells but uniform staining of M124/D125G-infected cells involving both nuclear and cytoplasmic regions (differentiated by DAPI staining of the nuclei). The comparative staining patterns could be interpreted to indicate that the full-length HK1 wild-type and M124/D125G mutant NS1 proteins were restricted primarily to the nucleus, whereas the polyclonal antibody detected additional staining of NS3 in the cytoplasm. This interpretation was supported by cellular fractionation into cytoplasm and nuclear fractions with detection of NS1 and NS3 proteins using the polyclonal antibody (Fig. 6b). This subcellular fractionation showed that cells infected with the NS1 wild-type and M124/D125G mutant had a similar and primarily nuclear distribution of full-length NS1 (when normalized to nuclear and cytoplasmic markers histone H3 and α-tubulin, respectively). This was especially true for the M124/D125G mutant that also expressed high levels of NS3 in both the cytoplasm and nucleus. These data therefore demonstrated the utility of mAb 3F5 for detecting full-length NS1 relative to the NS3 splice product that lacks this epitope, and furthermore indicated that NS1 protein and its variants possessing the 3F5 epitope can be discriminated from NS1 protein variants and deletion mutants that lack this epitope.

**The novel mAbs bind to highly conserved IAV epitopes**

This novel panel of defined NS1-specific mAbs will be useful for detecting NS1 proteins for diagnosis or characterization of infections. A screen of the >1800 non-redundant human IAV NS1 protein sequences available in the NCBI Influenza Virus Resource database (Bao et al., 2008) as of early November 2012, showed that three of these five epitopes are highly conserved across the majority of NS1 sequences. For example, the peptide sequences DCPF资格LDR and DCPF资格WHR, present in all six of our tested IAV strains and recognized by mAbs 5D6 and 10C7, are found in >95% of currently available human IAV NS1 sequences. Likewise, the DAPFL资格DR sequence, recognized by mAb 7D11, is also found in >95% of available sequences, and the peptides SPLPSLPGH and SPLPSFPGH, observed in all six of our tested strains and recognized by mAbs 3F5, 5F4 and 13D8, are found in >97% of currently available human IAV NS1 sequences. Given that the three-dimensional structure of NS1 has been determined for H5N1 NS1, and that NS1 has been shown to bind to several host proteins, our defined panel of mAbs could be used as ligand probes for binding variants and deletion mutants used to map regions of NS1 protein biological properties, such as was shown for the NS3 and NS1 proteins (Fig. 6). Similarly, the mAbs could be used to inhibit host interaction for assessment of the effects of blocking host factor binding to various sites in NS1.

**METHODS**

**Production of recombinant proteins.** Proteins were generated essentially as described by Sambrook et al. (1989). Native 6x His-tagged HK1 (H3N2) NS1 protein was expressed from a pET-17b (EMD Millipore) prokaryotic expression plasmid encoding NS1 in Escherichia coli. A cloned cDNA copy of the HK1 NS1 gene (GenBank accession no. CY030305) was amplified by PCR using NS1 forward (5’-ATTAGCAGTACGACTACCATACCATACCATACGATGTC-3’) and reverse (5’-CATGGAAATTCACGACTAC- ATCCTATAC-3’) primers. The PCR product was inserted into the Nhel and EcoRI sites of pET-17b. Soluble recombinant 6x His-tagged...
NS1 protein was expressed and purified by nickel affinity binding, as described previously (Ping et al., 2011).

A cloned cDNA copy of PR8 (H1N1) (GenBank accession no. EF190978) was amplified by PCR using the NS1 forward (5'-ATCCCCGGAATTCCCATGGATCCAAACAC-3') and reverse (5'-CGATGCGGCCGCTCAAACTTCTGACCTAAT-3') primers. The PCR product was inserted into the EcoRI and NotI sites of pGEX-5X-2 (GE Healthcare) under the control of the tac promoter, and the product, fused to GST, was then expressed in E. coli BL21(DE3) (Invitrogen). E. coli was grown in Luria–Bertani medium at 37°C until OD600 0.5 was reached; IPTG was then added to a final concentration of 0.1 mM to induce the cells, and the bacteria were incubated at room temperature for an additional 5 h. After harvesting the bacterial culture, proteins were extracted using B-PER II Bacterial Protein Extraction Reagent (Pierce). The GST–NS1 fusion protein was purified using a glutathione–agarose (Sigma) column.

**Mice immunization and mAb production.** Mouse immunizations were performed according to the guidelines of the Canadian Council on Animal Care. BALB/c female mice (6–8 weeks old) were immunized with 100 μl PBS containing 40 μg HK1 6His–NS1 and equal volumes of Titre-Max Gold adjuvant (Cedarlane Laboratories). A single boost of 50 μg 6His–NS1 per mouse was given 1 month after the first immunization. The spleen cells of immunized mice were fused with myeloma SP2/0 at a ratio of 4:1 (spleen:SP 2/0). The hybridomas were grown in RPMI 1640 (Gibco, Invitrogen) with 15% FBS and 20% mouse spleen-conditioned medium. Supernatants were screened after 13 days using 2.5% agarose, 100 μg/ml 5-bromo-4-chloro-3-indolyl–β-D-galactopyranoside (X-gal) and 2 mM IPTG in M9 minimal medium. Positive hybridomas were then cloned using limiting dilution and tested for NS1-reactive mAbs. One hybridoma secreting mAb 3F5 specific for NS1 was selected for further studies.

**Fig. 6.** Differential subcellular localization of the HK1 wild-type (wt) NS1 and recombinant HK1 M124I/D125G mutant NS3 proteins. (a) Mouse M1 cells were infected with the HK1 wild-type virus (HK1) or the NS1 mutant M124I/D125G (D125G) and fixed and stained in parallel with either the polyclonal anti-NS1 antibody that cross-reacts with the NS3 protein or with anti-NS1 mAb 3F5 specific for NS1. Cy3-conjugated secondary antibodies and DAPI were then applied to each set of cells. Representative confocal microscopy images are shown; magnification 63× (oil immersion). The bottommost HK1 wild-type row is an overexposure (OE) of the corresponding second row to demonstrate low-level reactivity to wild-type NS1 under these conditions relative to the abundance of NS1 and NS3 expression by the mutant. (b) Mouse M1 cells were infected (m.o.i. 2) with HK1 wild-type or recombinant HK M124I/D125G mutant, and at 16 h.p.i., the cells were lysed and differentially centrifuged to obtain whole-cell (W), nuclear (N) and cytosolic (C) fractions. The cell fractions were resuspended in SDS buffer and separated by SDS-PAGE, followed by Western blot analysis to detect the NS1 protein as well as loading markers α-tubulin and histone H3. A representative blot of two to three independent experiments is shown.
GST–NS1 of PR8 antigen by ELISA. The positive clones were screened again by ELISA and Western blotting, and were subcloned twice by the technique of limiting dilution (Harlow & Lane, 1988).

**Isotyping and purification of mAbs.** Isotyping was carried out with a mouse mAb isotyping kit (Amersham). mAbs were purified on protein G columns (GE Healthcare) after the hybridomas were grown in serum-free RPMI (Gibco) until death of the all the cells (~2 weeks). The supernatants were collected, passed through protein G columns, washed five times with PBS and eluted with glycine elution buffer (pH 2.7). The glycine was removed by dialysis in PBS and the concentrations of the purified antibodies were determined using a bicinchoninic acid assay (Pierce).

**Viruses and cell lines.** IAV strains PR8 (H1N1), B99 (H1N1), NCal (H1N1), NY55 (H3N2), HK1 (H3N2) and B10 (H3N2) (Table 1) were grown in 10-day-old embryonated chicken eggs. Viral stocks were titrated in MDCK cells by a standard plaque assay (Brown, 1990). Epithelial cells from canine kidney (MDCK), human lung (A549) and mouse kidney (MDI) were used to test the NS1 and NS3 proteins of various viruses. M1, MDCK and A549 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10 % FBS, 1 mM sodium pyruvate, 10 % (v/v) non-essential amino acids (Gibco, Invitrogen) and 2 mM L-glutamine.

**Immunoblotting.** M1, MDCK and A549 cells were infected with various IAVs at a m.o.i. of 1, 3 or 5 p.f.u. per cell. Cell lysis was obtained at various times (0–24 h p.i.) by lysing infected cells in 0.5 % NP-40 with protease inhibitor (Roche). Samples were mixed with an equal volume of 2 % SDS electrophoresis sample buffer and resolved in a 4–12 % gradient Novex NuPAGE SDS-PAGE Gel System (Invitrogen) and transferred to Immobilon-P PVDF membranes (Millipore). Equivalent aliquots of cell lysates were also heated and immobilized on Immobilon-P PVDF membranes for slot-blot (Hoefer PR 648) analysis. The membranes were treated with NS1 mAbs after blocking with 5 % skimmed milk. Blots were probed with HRP-linked anti-mouse polyclonal secondary antibody (Cell mAbs after blocking with 5 % skimmed milk. Blots were probed with HRP-linked anti-mouse polyclonal secondary antibody (Cell Signalling) and signals detected using enhanced chemiluminescence (in-house reagent). Images were obtained with an Alpha Innotech FluorChem Q Imaging System.

**Immunofluorescent microscopy.** MDCK cells were infected with IAV (m.o.i. 1 and 5) and harvested at various times (0 and 6–24 h p.i.). Cells were fixed with 3–4 % paraformaldehyde for 15 min, permeabilized with 0.1 % Triton X-100 for 5 min, blocked with 1 % BSA in PBS for 1.5 h and treated with each purified mAb in 1 % BSA/PBS overnight at 4 °C. Cells were washed four times with PBS and then treated with Alexa Fluor 546 (diluted 1:250; Invitrogen)- conjugated goat anti-mouse secondary antibodies in 1 % BSA/PBS for 1 h at room temperature, phalloidin 488 (diluted 1:200; Invitrogen) and DAPI (diluted 1:10,000; Invitrogen). Slides were washed three times with PBS, a drop of mounting medium was added (ProLong Gold; Invitrogen) to each spot and images were obtained with a Zeiss LSM710 laser-scanning microscope (Carl Zeiss MicroImaging), using 20 × and 40 × objectives.

**Confocal microscopy.** To observe the localization pattern of NS1 in infected cells, M1 cells were infected with recombinant HK1 wild-type, or the NS1 M124I/D125G mutant, at an m.o.i. of 3 in the absence of trophsin. At 16 h p.i., the cells were washed with PBS, fixed in 3.7 % formaldehyde for 10 min, washed with PBS and then permeabilized in 0.1 % Triton X-100 in PBS for 5 min. Following two washes in PBS, the cells were incubated with rabbit anti-HK1 NS1 polyclonal antibody (Selman et al., 2012) or mouse anti-NS1 mAb 3F5 overnight at 4 °C. The cells were washed twice with PBS and the slides were incubated with secondary Cy3-conjugated donkey anti-rabbit or donkey anti-mouse IgG, respectively (Jackson Immunoresearch Laboratories) for 2 h at room temperature. After two washes in PBS, the nuclei were stained by incubation with DAPI (300 ng ml⁻¹) in the dark for 15 min. Excess stain was removed by three washes with dH₂O. Cover slides were mounted with mounting medium (Dako) following the manufacturer’s protocol. The cells were viewed using a Zeiss LSM 510 META/AxiowVert 200 Confocal Microscope with a 63 × oil-immersion lens. Representative images were processed in parallel (GNU Image Manipulation Program 2.0).

**Nuclear and cytoplasmic fractionation of infected cells.** At 16 h p.i., confluent monolayers of M1 cells infected at an m.o.i. of 2 were washed once with ice-cold PBS and lysed with ice-cold NP-40 lysis buffer (50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 10 % glycerol, 1 % NP-40, 1 mM EDTA), and adherent cells were collected by scraping. The cells were fractionated as described previously (Suzuki et al., 2010). The whole cell, cytoplasmic and nuclear fractions were mixed with 4 × Laemmli sample buffer and boiled for 3 min prior to protein separation by SDS-PAGE. The immunoblots were probed for NS1 (polynomial rabbit anti-NS1), histone H3 (rabbit anti-histone H3, CT, pan, clone A35; Millipore) and α-tubulin (mouse anti-tubulin α; Sigma). Antibodies were detected by incubation with HRP-conjugated goat anti-rabbit or goat anti-mouse antibody (Sigma), followed by SuperSignal West Pico chemiluminescent substrate (Pierce, Thermo Fisher Scientific) and exposure to film.

**Epitope mapping, competitive ELISA and structural placement.** Aliquots of each purified mAb were submitted to Pepscan Therapeutics (The Netherlands) for linear overlapping peptide epitope mapping (Slootstra et al., 1996). Briefly, 216 15mer peptides were synthesized by Pepscan with 1 aa overlaps, each targeting the PR8 strain of NS1, each was bound to a Pepscan array, each array was reacted with each antibody and bound antibody was detected by ELISA with fluorescently labelled anti-mouse secondary antibody. Fluorescent intensities were recorded with a CCD camera and the intensities imported into Excel for graphing.

Competition between various epitopes was determined essentially as described by Harlow & Lane (1988). Briefly, Nunc 96-well plates were coated with 100 µl GST–PR8–NS1 (0.25 µg ml⁻¹). After overnight coating, the wells were washed three times with 0.05 % Tween 20 in PBS. The plates were blocked with 1 % BSA in PBS, washed and then treated with the various tested blocking antibodies (500 µg ml⁻¹) overnight at 4 °C. The plates were washed five times with 0.05 % Tween 20 in PBS and incubated with biotinylated mAb 8C7 (10 µg ml⁻¹) for 2 h. After washing three times with 0.05 % Tween 20 in PBS, the wells were treated with a 1:2000 dilution of ExtrAvidin E2636 for 1 h, washed five times with 0.05 % Tween 20 in PBS and developed with alkaline phosphatase/p-nitrophenyl phosphate. Absorbance was measured at 405 nm on a BioTek Synergy 4 plate reader.

The full-length NS1 protein of IAV strain H5N1 (Protein Data Bank 3F5T; Bornholdt & Prasad, 2008), the only full-length NS1 protein currently available in the Protein Data Bank, was imported into and manipulated with the molecular graphics program PyMOL version 1.5.0.4 (Schrödinger, LLC).

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