



RESEARCH ARTICLE

A NS1-binding monoclonal antibody interacts with two residues that are highly conserved in seasonal as well as newly emerged influenza A virus

Su Hui Catherine Teo^{1,†,‡}, Jian-Ping Wu^{1,†}, Chee-Keng Mok¹ and Yee-Joo Tan^{1,2,*}

¹Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, MD4, 5 Science Drive 2, Singapore 117545 and ²Institute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), 61 Biopolis Dr, Singapore 138673

*Correspondence author: Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, MD4, 5 Science Drive 2, Singapore 117545. Tel: +65 65163692; E-mail: yee.joo.tan@nuhs.edu.sg.

One sentence summary: Generation and characterization of a novel monoclonal antibody which binds to two highly conserved residues in NS1 of influenza A virus.

†equal contributing authors

Editor: Alfredo Garzino-Demo

‡Su Hui Catherine Teo, <http://orcid.org/0000-0002-1758-9515>

ABSTRACT

The non-structural protein 1 (NS1) of influenza A virus (IAV) is a multifunctional protein that antagonizes host antiviral responses, modulating virus pathogenesis. As such, it serves as a good target for research and diagnostic assay development. In this study, we have generated a novel monoclonal antibody (mAb) 19H9 and epitope mapping revealed that two residues, P85 and Y89, of NS1 are essential for interacting with this mAb. Furthermore, residues P85 and Y89 are found to be highly conserved across different IAV subtypes, namely seasonal H1N1 and H3N2, as well as the highly pathogenic H5N1 and H5N6 avian strains. Indeed, mAb 19H9 exhibits broad cross-reactivity with IAV strains of different subtypes. The binding of mAb 19H9 to residue Y89 was further confirmed by the abrogation of interaction between NS1 and p85 β . Additionally, mAb 19H9 also detected NS1 proteins expressed in IAV-infected cells, showing NS1 intracellular localization in the cytoplasm and nucleolus. To our knowledge, mAb 19H9 is the first murine mAb to bind at the juxtaposition between the N-terminal RNA-binding domain and C-terminal effector domain of NS1. It could serve as a useful research tool for studying the conformational plasticity and dynamic changes in NS1.

Keywords: non-structural protein 1 (NS1); monoclonal antibody 19H9; influenza A virus

INTRODUCTION

Influenza A virus (IAV) belongs to the *Orthomyxoviridae* family and is composed of eight single-stranded, negative-sense RNA segments. Of these, the NS segment is the smallest and

it encodes for the non-structural protein 1 (NS1) and nuclear export protein (NEP, also known as NS2). NS1 protein of IAV is a potent antagonist of the cellular antiviral interferon (IFN) response. Although it is not incorporated into the virus, it exerts multiple functions through the interaction with a large

Received: 3 December 2018; Accepted: 5 March 2019

© FEMS 2019. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

number of cellular components either in the cytoplasm or the nucleus. NS1 is able to inactivate the host antiviral interferon responses through a variety of mechanisms to facilitate replication during infection (Kochs, Garcia-Sastre and Martinez-Sobrido 2007). Although strain dependent, the general functions of NS1 include inhibiting the antiviral effects of 2'-5'-oligo (A) synthetase (OAS)/RNase L (Min and Krug 2006) and protein kinase R (PKR) (Li et al. 2006); activating phosphoinositide 3-kinase (PI3K) (Hale et al. 2006); inhibiting host gene expression by interacting with components of nuclear transport (Melen et al. 2007) and blocking mRNA export (Fortes, Beloso and Ortin 1994). Moreover, NS1 is also involved in suppressing adaptive immunity by attenuating human dendritic cell maturation and the capacity of dendritic cells to induce T-cell response (Fernandez-Sesma et al. 2006).

The NS1 protein is composed of 230–237 amino acids depending on the specific strain (Hale et al. 2008). NS1 has two well-characterized functional domains, namely, N-terminal RNA-binding domain (RBD) and C-terminal effector domain (ED). The RBD is composed of the first 73 amino acids of NS1, followed by the linker region (LR) at residues 74–88, and residues 88–202 constitute the ED (Hale 2014). NS1(RBD) contains the residues critical for the double stranded RNA (dsRNA) binding. NS1(ED) is responsible for interaction with various cellular proteins including PKR (Lu et al. 1995), the 30-kDa subunit of cleavage polyadenylation specificity factor 30 (CPSF30) (Nemeroff et al. 1998; Noah, Twu and Krug 2003), and some PDZ domain ligand proteins (Jackson et al. 2008).

Given that NS1 is highly expressed in infected cells and plays multiple functions, we have previously produced monoclonal antibodies (mAbs) binding to NS1 as tools for research and diagnostic assay development. Two of them were found to bind to NS1(RBD) and are able to bind different subtypes of IAV (Tan et al. 2010). In this study, we further generated and characterized another mAb, named as 19H9, which binds to two highly conserved residues P85 and Y89 in NS1.

MATERIALS AND METHODS

Cell lines

A549, 293T and MDCK cells were purchased from American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in Minimum Essential Medium (Gibco). 293T and MDCK cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen). Both media were supplemented with 10% fetal bovine serum (Hyclone), penicillin (10 000 units/ml)-streptomycin (10 mg/ml) solution (Sigma-Aldrich). All cell lines were maintained at 37°C with 5% CO₂.

Expression and purification of NS1

The NS1 fragment (residues 85–215) of A/Puerto Rico/8/1934(H1N1) was PCR amplified from a full-length NS1 gene (Accession NO.: ABD77680.1) and cloned into the pGEX-6P-1 vector (GE Healthcare). The GST-tagged wild-type and mutant NS1(ED) proteins were expressed and purified as described previously (Tan et al. 2010). The purified fusion proteins were dialyzed against phosphate-buffered saline (PBS) and the final concentration was determined using the Coomassie Plus protein assay reagent (Thermo Scientific). The purified proteins were concentrated to 3 mg/ml in a Centrprep-10 (Amicon) and stored in –20°C for subsequent assays.

Production and screening of hybridomas

Purified GST-NS1-85-215AA protein was used to immunize mice and generate hybridomas as described previously (Oh et al. 2010). MAb isotype was determined using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche). The mAb was purified using a HiTrap protein G HP affinity column (GE Healthcare) and was stored at –80°C. The purity of the mAb was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis. The concentration of the purified mAb was determined using the Coomassie Plus protein assay reagent (Thermo Scientific).

Immunofluorescence studies

For immunofluorescence analysis, MDCK cells on glass coverslips were fixed at 24 hr post-infection (hpi) in either ice-cold methanol for 10 min at –20°C or in 4% paraformaldehyde for 10 min at room temperature (RT) followed by permeabilization with 0.5% Triton X-100 (Sigma) for 5 min. Fixed cells were then blocked with PBS containing 10% fetal bovine serum for 30 min at RT. Cells were immunolabeled for 1 hr at RT with mAb 19H9 or mouse anti-nucleoprotein (NP; Millipore) and 45 min with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen) together with DAPI (Sigma), and mounting in ProLong® Gold Antifade Mountant (Molecular Probes). Images were acquired with Olympus CKX53 microscope using Olympus LCach N 20x/0.40 iPC objective lens and Olympus DP27 color camera with Olympus cellSens software. Each channel was collected separately, with images at 1024 × 1024 pixels.

Transient transfection, Western blot analysis and Immunoprecipitation (IP)

293T cells were seeded onto 6-well plates 24 hr prior to the transient transfection using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. NS1-expressing plasmids of different subtypes (H1N1, H3N2, H5N1 and H5N6) were used in this study. In addition, a series of N and C-terminal truncations and amino acid substitution mutants of H1N1-NS1 were generated by PCR using High Fidelity polymerase (Roche) and cloned into pXJ40-Myc expression vector. These mutant proteins were expressed by transient transfection. About 48 hr post-transfection, cells were harvested, spun down by centrifugation and washed with cold PBS twice. Cells were then resuspended in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40, 0.5% deoxycholic acid, 0.005% SDS and 1 mM phenylmethylsulfonyl fluoride] and subjected to freeze-thaw cycle 5 times. Clear supernatant containing the protein of interest was obtained by spinning down the cell lysate at 13 000 rpm at 4°C to remove the cell debris and further analyzed by Western blot analysis. Commercial antibodies against p85 β (Santa Cruz), GST (Santa Cruz), GAPDH (Santa Cruz), Myc (Santa Cruz) and NP (Millipore) were used. A home-made rabbit anti-NS1 polyclonal antibody (Wu et al. 2016) as well as the mAb 19H9 generated above were also used.

A total of 25 μ g of total cell lysates were resolved using electrophoresis on SDS-PAGE gels and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked in 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 hr at RT and incubated with primary antibodies at 4°C overnight. After the membrane was washed with TBST, it was incubated with a HRP-conjugated secondary antibody (Pierce) at RT for 1 hr. The membrane was then washed

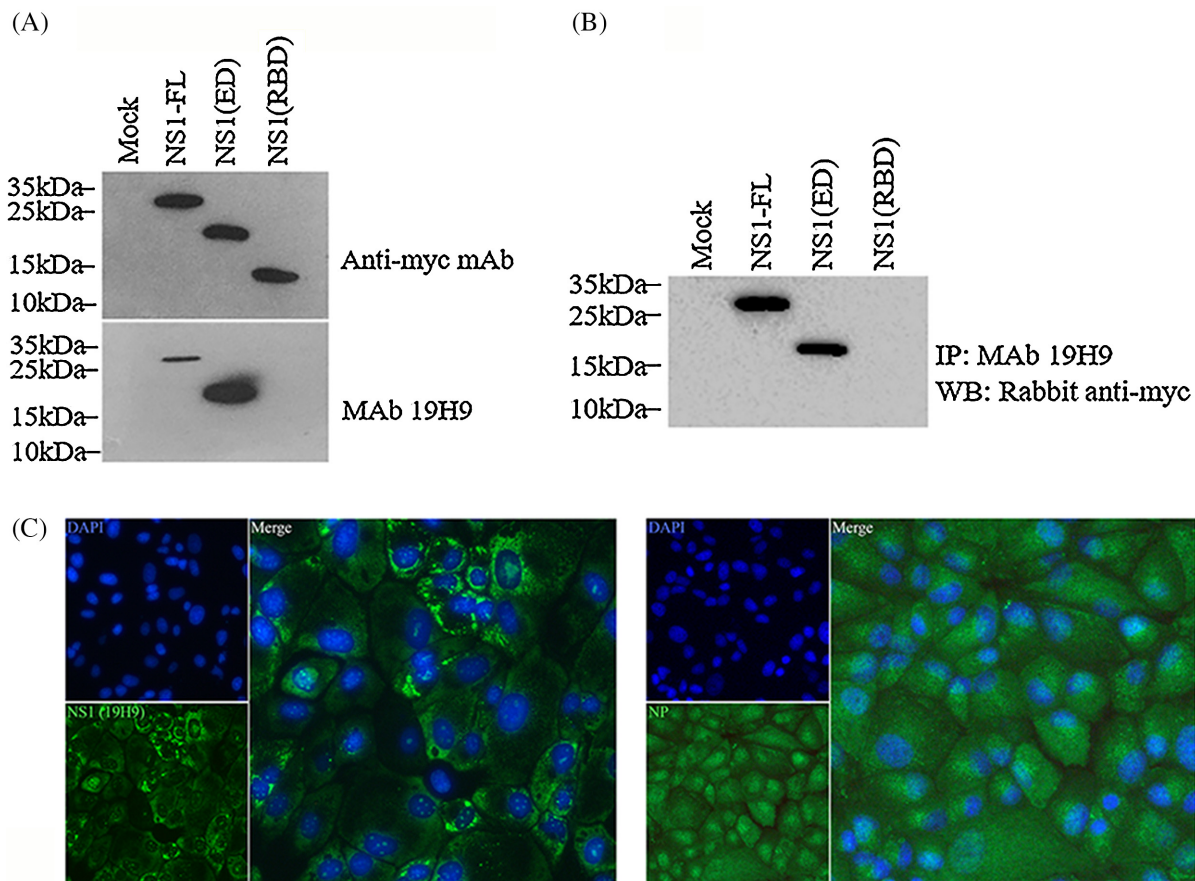


Figure 1. Binding of mAb 19H9 to NS1(ED). (A), Western blot analysis of NS1(RBD), NS1(ED) and full-length NS1 transfected cell lysate using either anti-Myc antibody (top panel), mAb 19H9 (bottom panel). (B), NS1(RBD), NS1(ED) and full-length NS1 transfected cell lysates were subjected to IP with mAb 19H9 and protein A agarose beads followed by detection of immunoprecipitated proteins using rabbit anti-Myc antibody in Western blot. Masses of molecular weight markers are indicated on the left in kDa. (C), MDCK cells were mock- or infected with rgPR8 H1N1 at an MOI of 1 for 24 hr, fixed and processed for immunofluorescence. The subcellular localization of NS1 and NP was detected by mAb 19H9 and mouse anti-NP mAb, respectively. Images were taken at magnification of 20x.

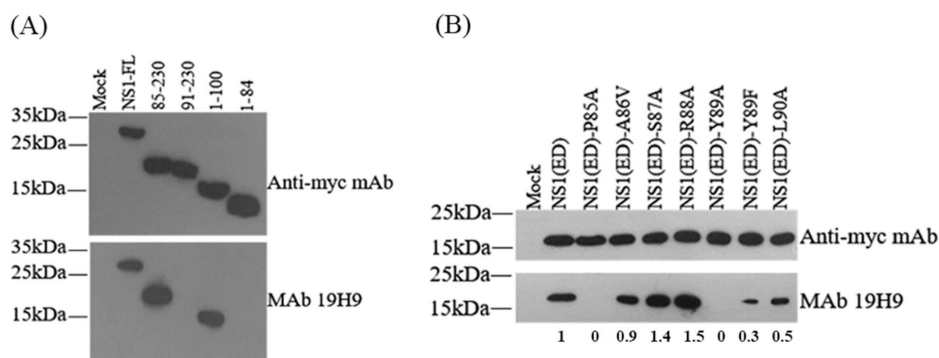


Figure 2. Epitope mapping of mAb 19H9. Western blot analysis of (A), a series of N-, C-terminal truncation and full-length NS1 protein and (B), single substitution mutants of NS1(ED). Western blot analysis was performed using either an anti-Myc antibody (upper panel) or mAb 19H9 (lower panel). Densitometric analysis of the band intensities for NS1(ED) using anti-myc and mAb 19H9, wherein expression of wild-type NS1(ED) was normalized to 1.

with TBST again and detected with enhanced chemiluminescence substrate (Pierce) using ChemiDoc™ MP Imaging System (Bio-Rad). For loading controls, the membrane was re-probed with rabbit anti-GAPDH (Santa Cruz Biotechnology) antibody overnight at 4°C, followed by secondary antibody and addition of enhanced chemiluminescence substrate (Pierce) for protein detection.

For IP, mAb 19H9 was used to pull down full-length NS1, NS1(RBD) and NS1(ED) in the cell lysates for 1 hr at 4°C, followed by the addition of Protein A beads (Roche) and then incubation at 4°C overnight. The beads were washed with RIPA buffer 3 times before subjected to Western blot analysis. Rabbit anti-Myc antibody was used as primary antibody to detect the presence of NS1 protein and goat anti-rabbit HRP-conjugated antibody (Pierce) was used as secondary antibody, followed by the

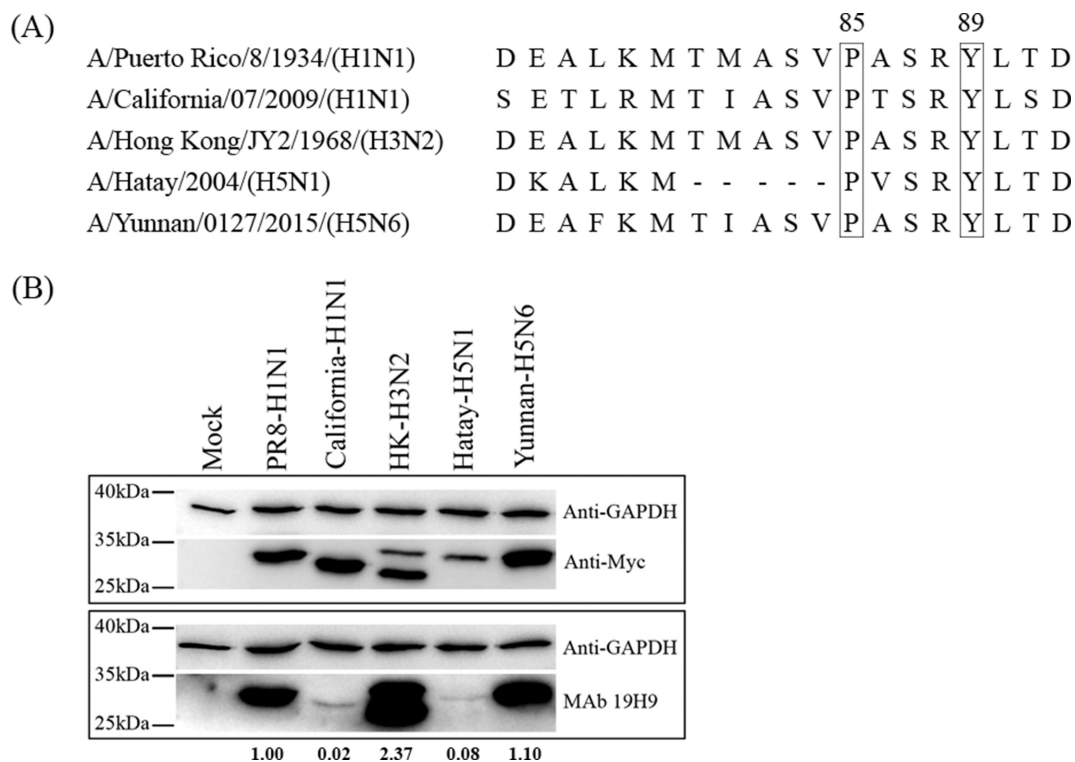


Figure 3. MAb 19H9 displays broad cross-reactivity with IAV strains of different subtypes. (A), Amino acid sequence alignment for residues 74–92 of NS1 protein of multiple subtypes of IAV as indicated. The mAb 19H9 binding sites at residues 85 and 89 are boxed. (B), Western blot analysis of NS1 of H1N1, H3N2, H5N1 and H5N6 expressed in 293T cells was performed using either anti-Myc antibody (upper panel) or mAb 19H9 (lower panel). GAPDH was used as a loading control. Densitometric analysis of the band intensities for NS1 using anti-myc and mAb 19H9, wherein expression of NS1 of the homologous PR8 virus was normalized to 1.

addition of enhanced chemiluminescence substrate (Pierce) for protein detection.

Generation of recombinant Influenza A/Puerto Rico/8/1934 virus from cloned DNA

Recombinant viruses were generated with pHW2000 reverse genetic system as described previously (Hoffmann *et al.* 2000). Residue 85 of NS1 was changed from P to A and residue 89 was changed from Y to F or A by PCR mutagenesis and the resulting DNA were inserted into pHW2000 vector. This plasmid was co-transfected with another seven pHW2000 plasmids encoding the other PR8 genomic RNAs into 293T cells. Two days post-transfection, culture supernatant was collected and used to infect MDCK cells. When cytopathic effect was visibly detected, culture supernatant was collected and used to infect naïve MDCK cells. Individual plaque was then amplified and viral titer was determined by plaque assay.

Plaque assay

About 90% confluent MDCK cells were adsorbed with serially diluted supernatants containing viruses for 1 hr at 37°C. The medium was discarded and the cells were rinsed using PBS. The cells were overlaid with 2 ml of DMEM supplemented by 0.3% agar and 2 µg/ml TPCk-trypsin (Thermo Scientific). After incubation at 37°C for 2 days, the cells were fixed using 10% formalin for 1 hr and stained using 0.1% crystal violet solution.

GST pull-down competition assay

About 5 µg GST-tagged NS1(ED) fusion proteins were mixed gently with 40 µl of 50% slurry of glutathione Sepharose 4B beads for

2 hr at 4°C. Beads were washed three times with binding buffer [20 mM Tris (pH 7.4), 0.1 mM EDTA and 100 mM NaCl] followed by incubation with 100 µg total lysates containing overexpressed p85β protein together with a serial dilution of mAb 19H9 or control antibody at 4°C overnight. The control antibody is a murine mAb 1A9 which binds to the spike protein of Severe Acute Respiratory Syndrome Coronavirus (Lip *et al.* 2006; Ng *et al.* 2014). The p85β protein was overexpressed in 293T cells via transient transfection as described above and the p85β expression plasmid (pCMV6-XL6-p85β) was purchased (Origene). Subsequently, beads were sedimented by centrifugation, washed three times with the washing buffer [0.5% NP40, 0.1 mM EDTA, 20 mM Tris, pH 7.4, 300 mM NaCl]. Laemmli sample buffer was added to the beads which were boiled to release all bound proteins. Western blot analysis was subsequently performed to detect for p85β and NS1(ED) proteins.

Quantification and statistical analysis

Densitometric analysis of band intensities for Western blots were performed using ImageJ. Two-tailed Student's t-test was applied to evaluate the statistical significance of differences measured from the data sets obtained in three independent experiments. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Binding of mAb 19H9 to H1N1-NS1 protein

The generation of mAb 19H9 was carried out using the recombinant GST-tagged NS1(ED) protein of A/Puerto Rico/8/1934(H1N1) virus, which was expressed in and purified from *E. coli*. MAb

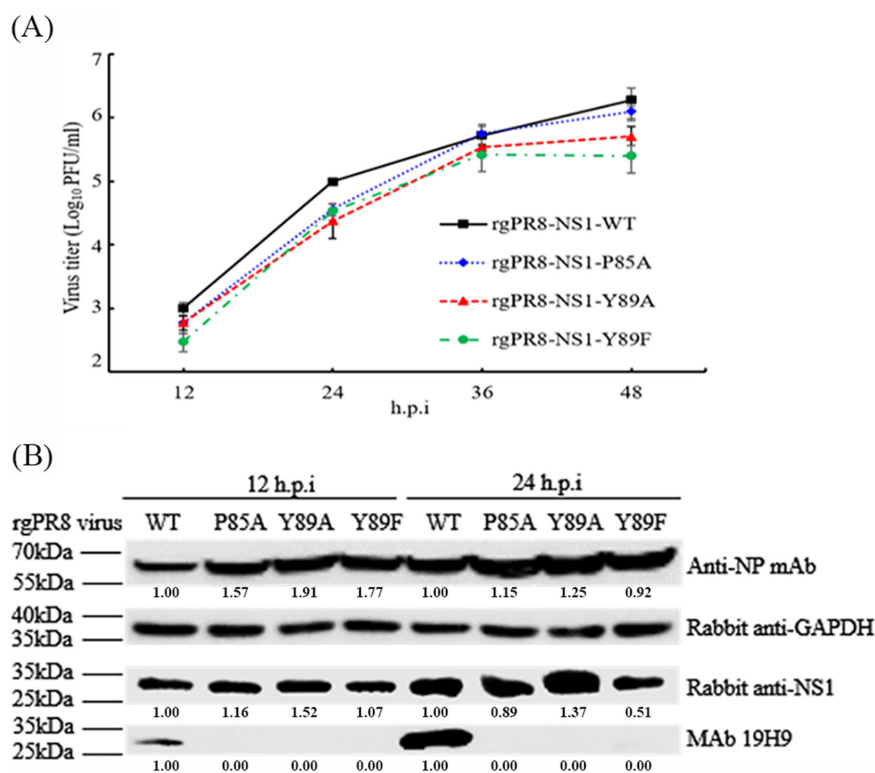


Figure 4. MAb 19H9 binds to P85 and Y89 of NS1 in recombinant IAV-infected cells. (A), Multi-cycle growth curves of rgPR8-NS1-WT, rgPR8-NS1-P85A, rgPR8-NS1-Y89A and rgPR8-NS1-Y89F recombinant viruses in A549 cells. Cells were infected at an MOI of 0.01 in duplicates, supernatants were collected at 12 hr interval over an infection period of 48 hr, and the virus titers were determined by plaque assays using MDCK cells. Mean titer values at each time point were plotted. (B), Western blot analysis of 293T cell lysates infected with recombinant viruses at an MOI of 1. Cell lysates were collected at 12 and 24 hpi and subjected to Western blot analysis using mAb 19H9, anti-NP, anti-GAPDH, anti-NS1 (Wu et al. 2016) antibodies. The experiments were repeated three times independently and a representative set of data is shown. Densitometric analysis of the band intensities for NP and NS1 using anti-NP, anti-NS1 and MAb 19H9, respectively, wherein expression of wild-type NS1 was normalized to 1 at each time point.

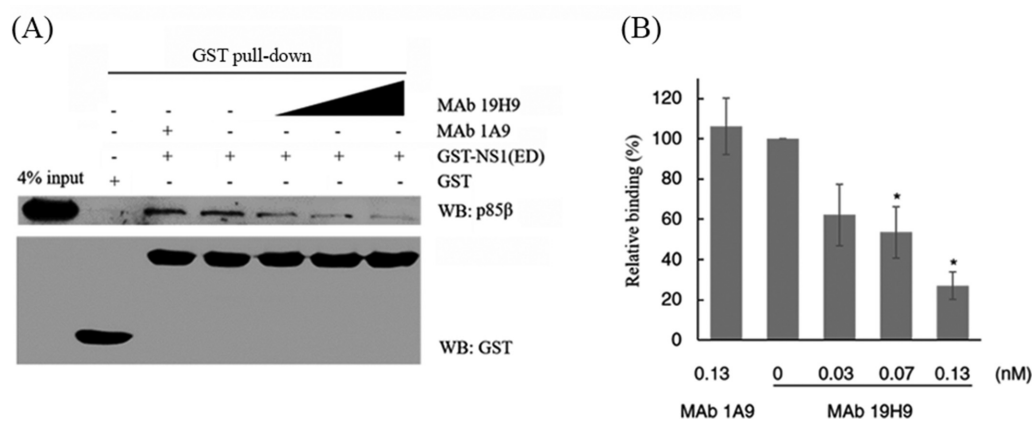


Figure 5. MAb 19H9 binds to residue Y89 of NS1 and blocks its interaction with p85 β . (A), Sepharose 4B beads bearing 5 μ g of GST (lane 2) or 5 μ g GST-NS1(ED) (lanes 3–7) were incubated with 100 μ g 293T cell lysates transiently transfected to overexpress p85 β protein together with indicated amount of control mAb 1A9 (lane 3) or mAb 19H9 (lanes 4–7) at 4°C for 2 hr. After washing, bound p85 β , GST and GST-NS1(ED) were probed by p85 β and GST antibodies, respectively. (B), The relative p85 β –NS1 interaction in the absence of 19H9 was arbitrarily set at 100%, which was used to normalize the relative binding in the presence of indicated concentrations of mAb 19H9 or control mAb 1A9.

19H9 was able to recognize the NS1(ED) upon initial screening using ELISA (data not shown). The isotype of mAb 19H9 was determined to be IgG2a, using IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche) according to the manufacturer's protocol. The Myc-tagged full-length NS1, NS1(RBD) and NS1(ED) proteins were expressed in 293T cells via transient transfection. As shown by Western blot analysis (Fig. 1A, bottom panel),

mAb 19H9 only detected full-length NS1 and NS1(ED), suggesting its specificity to ED. The expression level of NS1 proteins was verified using an anti-Myc antibody (Fig. 1A, top panel). Since Western blot analysis only detected denatured form of proteins, immunoprecipitation assay was also performed to determine whether mAb 19H9 could recognize NS1 under native condition. As shown in Fig. 1B, mAb 19H9 immunoprecipitated full-length

NS1 and NS1(ED) proteins in native state, while no binding was detected with NS1(RBD). Thus, mAb 19H9 is able to bind to both denatured and native forms of NS1 with specificity to the ED.

Furthermore, for spatial analysis of NS1 expressed in H1N1-infected cells using mAb 19H9, MDCK cells were mock- or infected with A/Puerto Rico/8/1934/(H1N1) (rgPR8) generated by reverse genetics at a multiplicity of infection (MOI) of 1 for 24 hr, fixed and processed for imaging analysis. Infection was determined by immunolabeling for another viral protein, NP and the expression of NP was observed in all the cells exhibiting nuclear and cytoplasmic localization. Figure 1C demonstrates that mAb 19H9 binds to NS1 expressed in H1N1-infected cells. In addition, NS1 displayed cytoplasmic as well as nucleolar localization. This results are consistent with previous data (Greenspan, Palese and Krystal 1988; Garaigorta, Falcon and Ortin 2005; Melen et al. 2007; Tsai et al. 2017) on the subcellular localization of NS1 as virus strains (Newby, Sabin and Pekosz 2007) and duration of infection (Melen et al. 2007) can determine NS1 localization pattern. Thus, mAb 19H9 could serve as a useful tool for studying NS1 cellular localization, especially in relation to its functions in the cytoplasm and nucleolus.

Epitope mapping reveals P85 and Y89 as two binding sites of mAb 19H9

To delineate the epitope of NS1 recognized by mAb 19H9, a series of NS1 truncation mutants consisting of residues 1–84, 1–100, 91–230 and 85–230 were generated and their expression levels in transiently transfected 293T cells were verified using an anti-Myc antibody (Fig. 2A, upper panel). Western blot results showed that the minimal epitope region lies in the amino acids 85–90 (⁸⁵PASRYL⁹⁰) as mAb 19H9 could only bind NS1 fragments containing this motif (Fig. 2A, lower panel).

Next, alanine scanning was used to determine the relative contribution of each of these residues to the interaction with mAb 19H9. NS1(ED) mutants containing a single alanine substitution (at residue 85, 87, 88, 89, 90, in the case of residues 86 and 89, A86V mutant and a more conservative Y89F mutant were also constructed) were constructed and tested for the binding of mAb 19H9. The expression levels of these mutants in transiently transfected 293T cells were verified using an anti-Myc antibody (Fig. 2B, upper panel). As illustrated in Fig. 2B (lower panel), the binding of mAb 19H9 to alanine mutants at position 85 or 89 was totally abolished, while the binding to the more conservative Y89F mutant was significantly reduced. On the other hand, single substitution at residues 86, 87, 88 and 90 did not affect binding of mAb 19H9.

mAb 19H9 displays broad cross-reactivity with IAV strains of different subtypes

Fig. 2 shows that residues P85 and Y89 of NS1 are essential for the binding of mAb 19H9. Amino acid sequence alignment revealed that these two residues are highly conserved among the seasonal H1N1 and H3N2, as well as highly pathogenic H5N1 and H5N6 avian IAV strains (Fig. 3A). In order to investigate whether mAb 19H9 interacts with the two residues conserved across different subtypes, NS1 of H1N1 and H3N2, as well as H5N1 and H5N6 was expressed in 293T cells via transient transfection. Indeed, mAb 19H9 was able to detect NS1 of different subtypes (Fig. 3B). Thus, mAb 19H9 not only binds to NS1 of the homologous PR8 virus but also cross-reacts with NS1 of different IAV subtypes.

mAb 19H9 binds to P85 and Y89 of NS1 in recombinant IAV-infected cells

Besides testing the binding of mAb 19H9 to NS1 protein over-expressed in 293T cells, recombinant viruses with various NS1 mutations (rgPR8-NS1-P85A, rgPR8-NS1-Y89A and rgPR8-NS1-Y89F) were generated so that binding of mAb 19H9 to NS1 expressed in infected cells can be assessed. The replication potential of these recombinant viruses was first assessed by comparing their multi-step growth kinetics at 12, 24, 36 and 48 hpi in A549 cells infected with the viruses at an MOI of 0.01. As shown in Fig. 4A, all mutant viruses displayed marginally slower growth kinetics as compared to the wild-type virus, however, the difference was not statistically different.

In the single-step growth kinetics assay, 293T cells were infected with wild-type and mutant viruses at an MOI of 1, harvested at 12 and 24 hpi and cell lysates were subjected to Western blot analysis to assess the level of viral protein synthesis. As shown in Fig. 4B, single mutation at either residue P85 or Y89 of NS1 did not affect the rate of viral protein synthesis in single-cycle replication, since the expression levels of viral NP in mutant viruses were comparable to that of the wild-type virus at both time points. Similarly, the level of NS1, as determined by using a rabbit anti-NS1 polyclonal antibody (Wu et al. 2016), was also comparable for all four viruses. However, mAb 19H9 only bound strongly to rgPR8-NS1-WT virus and did not bind the three mutant viruses. Consistent with the Western blot results obtained using recombinantly expressed NS1(ED) fragments, these results suggested that residues P85 and Y89 are required for mAb 19H9 binding to viral NS1 expressed in infected cells during viral infection.

mAb 19H9 binds to residue Y89 of NS1 and blocks its interaction with p85 β

It has been shown that NS1 binds specifically and directly to the p85 β regulatory subunit of PI3K to activate the PI3K/Akt pathway, which in turn supports efficient virus replication (Ehrhardt et al. 2007). Structural study has revealed that residue Y89 of NS1 is positioned in the binding interface of NS1-p85 β complex and the conservative substitution at this residue (Y89F) disrupts this interaction (Hale et al. 2010). Since Y89 is also critical for the binding of mAb 19H9 to NS1, an *in vitro* GST pull-down competition assay was performed to investigate the effect of mAb 19H9 on the interaction between NS1 and p85 β . Bacterially expressed and purified GST (negative control) and GST-NS1(ED) proteins were incubated with glutathione Sepharose 4B beads followed by incubation, in the presence of mAb 19H9 or control antibody, with total cell lysates containing p85 β protein expressed by transient transfection. The amount of p85 β protein bound to GST or GST-NS1(ED) was detected by Western blot using antibodies specific to the p85 β subunit. As shown in Fig. 5A, p85 β specifically bound to NS1(ED) instead of GST, since GST alone failed to pull down the p85 β protein. Furthermore, mAb 19H9 inhibited the interaction between p85 β and NS1(ED) in a dose-dependent manner (Fig. 5A), implying that the binding of mAb 19H9 and p85 β to NS1(ED) is competitive. Quantification by densitometry showed that there was a significant reduction in the interaction between p85 β and GST-NS1(ED) when mAb 19H9 was used at 0.07 nM and 0.13 nM (Fig. 5B). On the other hand, the presence of 0.13 nM of control antibody (mAb 1A9) showed no effect on the interaction between p85 β and GST-NS1(ED). This result shows that mAb 19H9 binds to residue Y89 of NS1 and blocks its interaction with p85 β .

CONCLUSION

As mAbs targeting NS1 can be used for research and diagnostic assay development, multiple hybridomas have been generated to produce anti-NS1 antibodies. For those mAbs with epitope mapped, all of them have been shown to bind to motifs in either NS1(RBD) or NS1(ED) (Tan et al. 2010; He et al. 2013; Rahim et al. 2013; Wen et al. 2015; Sun et al. 2016). In this study, we have generated a novel mAb 19H9 and found that it interacts with two residues, P85 and Y89, of NS1 that are highly conserved in seasonal as well as newly emerged highly pathogenic avian IAV strains. Consistently, mAb 19H9 did not bind to rgPR8 mutant viruses carrying substitution at either of these positions. To our knowledge, mAb 19H9 is the first murine mAb binding at the juxtaposition between the RBD and ED of NS1. It could serve as a useful research tool for studying the conformational plasticity and dynamic changes in NS1. Interestingly, a fully human single chain antibody fragment (huScFv clone 10) was isolated from a human ScFv phage display library and found to bind to residues 75–90 of NS1 (A/duck/Thailand/144/2005(H5N1)) (Yodsheewan et al. 2013). The overlap between the epitopes of mAb 19H9 and huScFv clone 10 suggests that the juxtaposition between the RBD and ED of NS1 is immunogenic.

The residue Y89 is part of the ED of NS1 and previous study has demonstrated that it is important for NS1's interaction with the p85 β subunit of the PI3K complex (Hale et al. 2010). Indeed, this study has shown that the interaction between NS1(ED) and p85 β was abrogated by the binding of mAb 19H9 to Y89 of NS1. On the other hand, P85 is found in the LR between the RBD and ED. While the exact role of the LR in viral replication has not been defined, five amino acids deletion (residues 80–84) is found to be implicated in the high virulence of the H5N1 strains like A/Vietnam/1203/2004/(H5N1) (Long et al. 2008). In addition, structural analysis showed that the NS1(ED) dimer in the full-length NS1 of A/Vietnam/1203/2004/(H5N1) exhibited significant difference from its structure when solved as an isolated domain (Bornholdt and Prasad 2008). Structural analysis of the full length H6N6-NS1 and a mutant H6N6-NS1 Δ 80–84 further revealed that the LR influences the orientation of NS1(ED) with respect to NS1(RBD) and thus plays an important role in determining the quaternary structure of NS1 (Carrillo et al. 2014).

The LR could be the basis for strain-specific functions associated with NS1 protein during IAV infection, possibly by determining the protein's correct subcellular localization. Using mAb 19H9, NS1 displayed cytoplasmic as well as nucleolar localization. As such, it could be used to study NS1 temporal distribution, and its interactions with host nucleolar or cytoplasmic proteins, during the course of virus infection. The interaction of mAb 19H9 with the LR of NS1 could potentially be useful in investigating the less well defined functions of this region.

ACKNOWLEDGMENTS

The authors declare that there is no conflict of interest regarding the publication of this article. This work was supported by the Singapore Ministry of Health's National Medical Research Council under its NMRC-CBRG scheme [grant no. NMRC/CBRG/0017/2012] as well as the National Research Foundation, Singapore, under its NRF-NSFC joint research grant call (Emerging Infectious Diseases) [grant no. NRF2016NRF-NSFC002-016].

Conflicts of interest. None declared.

REFERENCES

- Bornholdt ZA, Prasad BV. X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus. *Nature* 2008;**456**:985–8.
- Carrillo B, Choi JM, Bornholdt ZA et al. The influenza A virus protein NS1 displays structural polymorphism. *J Virol* 2014;**88**:4113–22.
- Ehrhardt C, Wolff T, Pleschka S et al. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J Virol* 2007;**81**:3058–67.
- Fernandez-Sesma A, Svetlana M, Ebersole Barbara J et al. Influenza virus evades innate and adaptive immunity via the NS1 protein. *J Virol* 2006;**80**:6295–304.
- Fortes P, Beloso A, Ortin J. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J* 1994;**13**:704–12.
- Garaigorta U, Falcon AM, Ortin J. Genetic analysis of influenza virus NS1 gene: a temperature-sensitive mutant shows defective formation of virus particles. *J Virol* 2005;**79**:15246–57.
- Greenspan D, Palese P, Krystal M. Two nuclear location signals in the influenza virus NS1 nonstructural protein. *J Virol* 1988;**62**:3020–6.
- Hale BG. Conformational plasticity of the influenza A virus NS1 protein. *J Gen Virol* 2014;**95**:2099–105.
- Hale BG, Jackson D, Chen YH et al. Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. *Proc Natl Acad Sci USA* 2006;**103**:14194–9.
- Hale BG, Kerry Philip S, Jackson D et al. Structural insights into phosphoinositide 3-kinase activation by the influenza A virus NS1 protein. *Proc Natl Acad Sci USA* 2010;**107**:1954–9.
- Hale BG, Randall RE, Ortin J et al. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol* 2008;**89**:2359–76.
- He JL, Hsieh MS, Chiu YC et al. Preparation of monoclonal antibodies against poor immunogenic avian influenza virus proteins. *J Immunol Methods* 2013;**387**:43–50.
- Hoffmann E, Neumann G, Kawaoka Y et al. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci USA* 2000;**97**:6108–13.
- Jackson D, Hossain MJ, Hickman D et al. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. *Proc Natl Acad Sci USA* 2008;**105**:4381–6.
- Kochs G, Garcia-Sastre A, Martinez-Sobrido L. Multiple anti-interferon actions of the influenza A virus NS1 protein. *J Virol* 2007;**81**:7011–21.
- Li S, Min JY, Krug RM et al. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 2006;**349**:13–21.
- Lip KM, Shuo S, Xiaoming Y et al. Monoclonal antibodies targeting the HR2 domain and the region immediately upstream of the HR2 of the S protein neutralize in vitro infection of severe acute respiratory syndrome coronavirus. *J Virol* 2006;**80**:941–50.
- Long JX, Peng DX, Liu YL et al. Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral non-structural gene. *Virus Genes* 2008;**36**:471–8.
- Lu Y, Wambach M, Katze MG et al. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. *Virology* 1995;**214**:222–8.

- Melen K, Kinnunen L, Fagerlund R *et al.* Nuclear and nucleolar targeting of influenza A virus NS1 protein: striking differences between different virus subtypes. *J Virol* 2007;**81**:5995–6006.
- Min JY, Krug RM. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc Natl Acad Sci USA* 2006;**103**:7100–5.
- Nemeroff ME, Barabino SM, Li Y *et al.* Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3' end formation of cellular pre-mRNAs. *Mol Cell* 1998;**1**:991–1000.
- Newby CM, Sabin L, Pekosz A. The RNA binding domain of influenza A virus NS1 protein affects secretion of tumor necrosis factor alpha, interleukin-6, and interferon in primary murine tracheal epithelial cells. *J Virol* 2007;**81**:9469–80.
- Ng OW, Choong-Tat K, Sau-Wai LC *et al.* Substitution at aspartic acid 1128 in the SARS coronavirus spike glycoprotein mediates escape from a S2 domain-targeting neutralizing monoclonal antibody. *PLoS One* 2014;**9**:e102415.
- Noah DL, Twu KY, Krug RM. Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAs. *Virology* 2003;**307**:386–95.
- Oh HL, Akerström S, Shen S *et al.* An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. *J Virol* 2010;**84**:8275–86.
- Rahim MN, Selman M, Sauder PJ *et al.* Generation and characterization of a new panel of broadly reactive anti-NS1 mAbs for detection of influenza A virus. *J Gen Virol* 2013;**94**:593–605.
- Sun J, Xiurong W, Xuexia W *et al.* Identification of a highly conserved epitope on avian influenza virus non-structural protein 1 using a peptide microarray. *PLoS One* 2016;**11**:e0149868.
- Tan Z, Akerstrom S, Wee BY *et al.* A new panel of NS1 antibodies for easy detection and titration of influenza A virus. *J Med Virol* 2010;**82**:467–75.
- Tsai CF, Hsin-Yi L, Wei-Li H *et al.* The novel mitochondria localization of influenza A virus NS1 visualized by FLAsH labeling. *FEBS Open Bio* 2017;**7**:1960–71.
- Wen X, Jiashan S, Xiurong W *et al.* Identification of a novel linear epitope on the NS1 protein of avian influenza virus. *BMC Microbiol* 2015;**15**:168.
- Wu J, Mok CK, Chow VT *et al.* Biochemical and structural characterization of the interface mediating interaction between the influenza A virus non-structural protein-1 and a monoclonal antibody. *Sci Rep* 2016;**6**:33382.
- Yodsheewan R, Maneewatch S, Srimanote P *et al.* Human monoclonal ScFv specific to NS1 protein inhibits replication of influenza viruses across types and subtypes. *Antiviral Res* 2013;**100**:226–37.