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# Human monoclonal ScFv specific to NS1 protein inhibits replication of influenza viruses across types and subtypes

Rungrueang Yodsheewan [a], Santi Maneewatch [b], Potjanee Srimanote [c], Kanyarat Thueng-in [d,1], Thaweesak Songserm [e], Fonthip Dong-din-on [f], Kunan Bangphoomi [g], Nitat Sookrung [h], Kiattawee Choowongkomon [g], Wanpen Chaicumpa [d,*]

[a] Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[b] Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[c] Graduate Program in Biomedical Science, Faculty of Allied Health Sciences, Thammasat University, Pathumthani Province 12120, Thailand
[d] Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[e] Department of Veterinary Pathology, Faculty of Veterinary Medicine, Kasetsart University, Kam-paeng-saen Campus, Nakhon-Pathom Province, Thailand
[f] Center for Agriculture Biotechnology, Kasetsart University, Bangkok 10900, Thailand
[g] Department of Biochemistry, Faculty of Sciences, Kasetsart University, Bangkok 10900, Thailand
[h] Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

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ABSTRACT

Currently, there is a need of new anti-influenza agents that target influenza virus proteins other than ion channel M2 and neuraminidase. Non-structural protein-1 (NS1) is a highly conserved multifunctional protein which is indispensable for the virus replication cycle. In this study, fully human single chain antibody fragments (HuScFv) that bound specifically to recombinant and native NS1 were produced from three *huscfv*-phagemid transformed *Escherichia coli* clones (nos. 3, 10 and 11) selected from a human ScFv phage display library. Western blot analysis, mimotope searching/epitope identification, homology modeling/molecular docking and phage mimotope ELISA inhibition indicated that HuScFv of clone no. 3 reacted with NS1 R domain important for host innate immunity suppression; HuScFv of clone nos. 10 and 11 bound to E domain sites necessary for NS1 binding to the host eIF4GI and CPSF30, respectively. The HuScFv of all clones could enter the influenza virus infected cells and interfered with the NS1 activities leading to replication inhibition of viruses belonging to various heterologous A subtypes and type B by 2–64-fold as semi-quantified by hemagglutination assay. Influenza virus infected cells treated with representative HuScFv (clone 10) had up-expression of IRF3 and IFN-β genes by 14.75 and 4.95-fold, respectively, in comparison with the controls, indicating that the antibodies could restore the host innate immune response. The fully human single chain antibodies have high potential for developing further as a safe (adjunctive) therapeutic agent for mitigating, if not abrogating, severe symptoms of influenza.


## 1. Introduction

At present, there are only two families of FDA-approved drugs for treatment of influenza, i.e., blockers of the polymeric M2 ion channel activity (amantadine and rimantadine) and neuraminidase (NA) inhibitors (oseltamivir, zanamivir and peramivir) which prevent viral release and spread. These drugs must be taken early in the course of the infection in order to expect high response rate. Drug resistant influenza viruses have increasingly emerged (McKimm-Breschkin, 2000; Bright et al., 2006; Deyde et al., 2007; Hurt et al., 2004, 2006; Renaud et al., 2011; Tang et al., 2008). Thus, new agents that target other influenza virus proteins and can tolerate the antigenic variation of the viruses should be sought.

Non-structural protein-1 (NS1) is a multifunctional and conserved protein of influenza viruses (Hale et al., 2008a). It is produced at the initial phase of the infection for suppressing and evading the host immune responses and enhancing the viral replication (Hale et al., 2008a; Kim and Samal, 2010; Lin et al., 2007). Each molecule of the NS1 comprises of 200–237 amino acids depending upon strains (Suarez and Perdue, 1998; Bornholdt and Prasad, 2006; Palese and Shaw, 2007; Suwannakhon et al., 2008).

* Corresponding author. Address: Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok-noi, Bangkok 10700, Thailand. Tel.: +66 2 4196497; fax: +66 2 4196491.
*E-mail address:* wanpen.cha@mahidol.ac.th (W. Chaicumpa).
[1] Present address: Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkhen Campus, Bangkok 10900, Thailand.

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The N-terminal domain (residues 1–73) is a double stranded (ds)-RNA binding (R) domain which functions in limiting host interferon (IFN) $\alpha/\beta$ production. The NS1 inhibits the cellular 2′,5′ oligoadenylate synthetase (OAS) activation by competing for the viral dsRNA (Min and Krug, 2006) and prevents the viral RNA from the OAS dependent RNase-L degradation. Because the OAS is inactive, the RNase L is in latency and refractory to the host IFN (Silverman, 2007). The R domain inhibits serine/threonine protein kinase R (PKR) activity, also by dsRNA competitive binding which prevents apoptosis of the influenza virus infected host cells through the PKR-signaling pathway (Min et al., 2007). The NS1 C-terminal (residues 74 to the end of the C terminus) is an effector (E) domain which interferes with PI3K/Akt pathway and promotes the host cell survival for viral replication and propagation (Hale et al., 2006, 2008b). The domain binds directly to cleavage and polyadenylation specific factor (CPSF30) and poly-A-binding protein-II (PABPII) causing inhibition of cellular pre-mRNA processing (elongation of poly-A tail is halted); therefore cellular protein synthesis is bias towards the viral benefit (Nemeroff et al., 1998; Chen et al., 1999). The E domain interact with eIF4GI and also poly-A-binding protein-I (PABPI) recruiting them to the 5′ end of the viral mRNA for enhancing the viral translation (Aragon et al., 2000; Burgui et al., 2003). Nascent NS1 protein can block active site of nucleolin and prevents nuclear export of the host mRNAs (Ong and Chan, 2012). Besides, the NS1 protein interferes with dendritic cell (DC) maturation and migration by regulating the relevant gene expressions which consequently causes the DC inability to stimulate IFN-$\gamma$ secretion from Th1 cells (Fernandez-Sesma et al., 2006). Therefore, agent that interferes with the influenza virus NS1 activities should result in restoring the host anti-viral activities, particularly the IFN based-innate immunity, as well as limiting the virus replication. In this study, human single chain variable antibody fragments (HuScFv) that bound specifically to the R and E domains of the NS1 protein were produced. The NS1 specific-HuScFv interfered with the replication across types and subtypes of influenza viruses.

## 2. Materials and methods

### 2.1. Viruses

Influenza viruses used in this study are listed in Table 1. Sendai virus was ATCC strain 52 (Fushimi). The viruses were propagated in either 8–10 days old embryonated eggs or Mardin-Darby canine kidney (MDCK) cells. For the latter, the MDCK cells inoculated with seed virus were grown in Dulbeco′s Modified Eagle Medium (DMEM) (Gibco) supplemented with 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% heat inactivated fetal bovine serum (Hyclone, Thermo Scientific) at 37 °C in 5% $CO_2$ atmosphere.

### 2.2. Production of recombinant NS1 proteins

Full length recombinant NS1 and R and E domain proteins were produced as described in Supplemental Materials and Methods 1.

### 2.3. Selection of phage clones that bound to recombinant NS1 and production of HuScFv

The method details are given in Supplemental Materials and Methods 2.

### 2.4. ELISAs

HuScFv expressed from individual *huscfv*-positive HB2151 *Escherichia coli* clones were tested for reactivity to recombinant F-NS1 by indirect and dot ELISAs (Kulkeaw et al., 2009) using lysate of original HB2151 *E. coli* and BSA as negative HuScFv and antigen controls, respectively.

### 2.5. Western blot analysis for determining NS1 domain bound by HuScFv

The recombinant F-NS1, R and E proteins were individually subjected to 12% SDS–PAGE and Western blot analysis as described previously (Thueng-in et al., 2012).

### 2.6. Virus neutralization tests

The virus (A/duck/Thailand-Nong-khai/KU-56/2007 H5N1) was either mixed with HuScFv before adding to the cells (test a) or added directly into the MDCK cells in the culture wells (test b) at MOI 0.5 using diluted lysate of pET32c$^+$ transformed BL21 (DE3) *E. coli* mixed with virus as control (c). Virus adsorption and cellular entry was allowed for 2 h at 37 °C in 5% $CO_2$ atmosphere. The fluid in each well was aspirated; the cells were washed gently with plain DMEM and the cells of tests a and b were replenished with serum supplemented DMEM (150 μl) containing 2 μg of HuScFv while the control cells (c) were added with diluted lysate of pET32c$^+$ transformed BL21 (DE3) *E. coli* which was used as negative HuScFv control. The plate was incubated at 37 °C in 5% $CO_2$. Every 2 h thereafter until 24 h, the medium in triplicate wells of each treatment was removed and the numbers of virus plaques in the cells were enumerated by using plaque assay. The virus infected cells

**Table 1**
Influenza viruses used in this study.

| Name | Subtype | Clade | Subclade | Reference, GenBank accession no. and/or relevant information |
|---|---|---|---|---|
| A/duck/Thailand/144/2005 | H5N1 | 1 | | Songserm et al. (2006) |
| A/dog/Thailand-Suphanburi/KU-08/2004 | H5N1 | 1 | | Isolated from moribund dog in Suphanburi province, central Thailand (GenBank accession no. DQ530170-**7**) |
| A/duck/Thailand-Nong-Khai/KU-56/2007 | H5N1 | 2 | 3 | Isolated from duck in Nong-Khai province, northeastern Thailand (GenBank accession no. EU221249-**56**) |
| A/swine/Iowa-15/30 | H1N1 | | | M33046 |
| A/Thailand/CU-68/2006 | Seasonal H1N1 | | | Isolated from infected patient of Chulalongkorn Hospital, Bangkok, Thailand in 2006 (GenBank accession no. FJ912931-**6**) |
| A/CTB/32/2011 (A/California/7/2009-like virus) | H1N1 Vaccine-like strain | | | GenBank accession no. FJ969536-**40** |
| A/swine/Thailand/KU-21/2004 | H3N2 | | | Isolated from infected pig in Thailand in 2004 |
| A/SRTN/23/2011 (A/Perth/16/2009-like virus) | H3N2 | | | GenBank accession no. GQ293081-**2** |
| B/TAK/42/2011 (B/Brisbane/60/2008-like virus) | Vaccine-like strain | | | GenBank accession no. FJ766841-**2** |

in each well were washed with PBS, fixed with 200 μl of absolute methanol overnight, washed again with PBS, permeabilized with 1% Triton X-100 (100 μl) for 30 min and blocked with 3% BSA (200 μl) for 1 h. Mouse polyclonal anti-M2 (primary antibody) was added and incubated. Goat anti-mouse immunoglobulin-alkaline phosphatase (AP) conjugate and BCIP/NBT substrate were used for revealing the virus plaques. The numbers of plaque at different time points were compared between different treatment groups.

Alternatively, influenza viruses (various strains of A subtypes and type B) and Sendai virus (control virus) were mixed with HuScFv/irrelevant HuScFv/lysate of original BL21 (DE3) *E. coli* and immediately added separately to MDCK cell monolayer grown in DMEM in triplicate wells of a 96 well tissue culture plate. The virus entry was allowed as above and the infected cells were grown in serum supplemented DMEM (150 μl) containing 2 μg of individual HuScFv, irrelevant HuScFv specific to puffer tetrodotoxin (Chulanetra et al., 2012) or diluted lysate of pET32c$^+$ transformed BL21 (DE3) *E. coli* and incubated further for 22 h. The spent culture fluids in all wells were collected separately and subjected to hemagglutination assay for semi-quantification of the released viruses as described previously (Maneewatch et al., 2009). Cells in the wells infected with type A and Sendai viruses were subjected to cell-ELISA for determining the amounts of the intracellular influenza virus NS1 and M2 and Sendai virus, respectively. Cells in the wells infected with type B influenza virus were subjected to comparative real-time PCR (qPCR) for determining the amounts of intracellular viral RNA coding for hemagglutinin (HA) and neuraminidase (NA). Sendai virus N protein in the virus infected cells was also determined by the qPCR. Three independent experiments were performed.

### 2.7. Hemagglutination assay and cell ELISA

For hemagglutination assay, samples containing influenza viruses were two-fold serially diluted with PBS in a V-bottom microplate (25 μl/well). To each well, 25 μl each of PBS and 1% goose erythrocytes in PBS were added and mixed. The plate was kept at 25 °C until the red blood cells in the control wells (wells containing only 50 μl PBS and 25 μl red blood cells) set as a button at the bottom of the well (negative hemagglutination). The hemagglutination titer (the highest virus dilution that caused complete agglutination of the erythrocytes) of each sample was determined.

For the ELISA, the cells infected with type A viruses and Sendai virus in individual wells were washed with PBS, fixed with 200 μl of absolute methanol overnight, washed again with PBS, permeabilized with 1% Triton X-100 (100 μl) for 30 min and blocked with 3% BSA (200 μl) for 1 h. For influenza virus antigens, mouse polyclonal anti-influenza NS1 or M2 (100 μl) was added and kept for 1 h then the cells were washed with PBST. Goat anti-mouse immunoglobulin-HRP conjugate (100 μl) was added to each well, kept for 1 h, washed and added with ABTS substrate. After 15 min, the fluids in all wells were placed appropriately in new microplate wells and $OD_{405nm}$ were determined. For Sendai virus detection, chicken polyclonal antibody to Sendai virus (Abcam, UK), goat anti-chicken immunoglobulin, rabbit anti-goat immunoglobulin-HRP and ABTS substrate were used.

### 2.8. Comparative real-time RT-PCR

After collecting the spent culture fluids in all wells, total RNA was extracted from the influenza B and Sendai virus infected cells by using RNeasy Mini Kit (Qiagen). The RNA of each sample was quantified by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Two pairs of primers specific to gene sequences coding for influenza B virus HA and NA (*HA*: forward 5'-ACGGATACACATCCCATGGGGCA-3′ and reverse 5′-TGGAGTTCATCCATGGCACCGC-3′; *NA*: 5′-TGCCCGGGCTCAACCTTTCAG-3′ and reverse 5′-CCCCCT GGTTGGGCTGCATAA-3′) were designed from B/Brisbane/60/2008 virus (GenBank accession no. FJ766841). Primer pair specific to Sendai virus nucleoprotein (N) gene (forward 5′-GTGACGGATACAGCCAAGGG-3′ and reverse 5′-ACTATCTCCACCCCAACCCC-3′) were designed from Sendai virus strain Z (GenBank accession no. AB753449.1). PCR master mix was prepared on ice with 2× Brilliant II SYBR® Green QRT-PCR Master Mix (Agilent), RT/RNase block enzyme, 200 nM of each primer and RNA solution (equal amount of total RNA) in a total of 12.5 μl. PCR was carried out using Rotor Gene (RG-3000) machine (Corbett Research, Australia) to compare the amounts of the RNA coding for influenza B virus HA and NA and Sendai virus N protein in the samples using GAPDH RNA for normalization. The qPCR conditions were: reverse transcription at 42 °C for 1 h, initial denaturation at 95 °C for 10 min and 40 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. To analyze dissociation curve, the following thermal profile was used: 95 °C for 1 min then ramped down to 55 °C (0.5 °C/s) and ramped up to 95 °C.

### 2.9. Effect of HuScFv on NS1 production by influenza virus

MDCK cell monolayer grown on glass cover slips in tissue culture wells were infected with influenza viruses in the presence of either NS1 specific-HuScFv or lysate of BL21 *E. coli* carrying pET32c$^+$ without the *huscfv* insert (negative HuScFv control). Viral entry was allowed for 2 h; the cells were washed and replenished with fresh medium containing the HuScFv or the negative HuScFv control for 22 h. After discarding the culture fluid, the cells on the cover slips were washed with PBS, fixed with methanol, permeabilized with 1% Triton X-100 (100 μl) for 30 min, blocked with 3% BSA (200 μl) for 1 h and washed again with PBST before adding with mouse polyclonal anti-NS1 antibody for 1 h. After washing, the cells were allowed to react with chicken anti-mouse immunoglobulin-Alexa Fluor 488 (Invitrogen) for 1 h, washed with PBST and subjected to confocal microscopy for determining the amounts of the native NS1 in the infected cells.

### 2.10. Detection of HuScFv, native NS1 and lipid raft in the influenza virus infected cells

MDCK cell monolayer grown on glass cover slips in tissue culture wells were infected with influenza viruses in the presence of NS1 specific-HuScFv. After allowing the viral entry for 2 h, the cells were washed and replenished with fresh medium containing the HuScFv/lysate of *E. coli* control for 4 h. Non-infected cells were included in the experiment. After discarding the culture fluids, the cells on the cover slips were washed with PBS, fixed, permeabilized, blocked for 1 h and washed again with PBST before adding with rabbit polyclonal anti-NS1 antibody and mouse monoclonal anti-6x-His tag and kept for 1 h. After washing, the cells were allowed to react with chicken anti-mouse immunoglobulin-Alexa Fluor 488, goat anti-rabbit immunoglobulin-Alexa Fluor 594 and cholera toxin B (CT-B) subunit-Alexa Fluor 647 (Invitrogen) for 1 h (the labeled CT-B bound to pentasaccharide chains of $G_{M1}$ gangliosides which located selectively in the cellular lipid raft). The stained cells were observed under 2.4 μm sectional laser confocal microscopy for HuScFv (green), the native NS1 (red) and the cellular lipid raft (blue) at different cellular layers.

### 2.11. Phage peptides (mimotopes) that bound to NS1 specific-HuScFv and NS1 residues bound by the HuScFv

Phage peptides (mimotopes) that bound to the NS1 specific-HuScFv were determined by using a phage display 12-mer peptide library (Ph.D.™-12 Phage Display Peptide Library, New England

Biolabs) (Sookrung et al., 2011). The method details are given in Supplemental Materials and Methods 3.

### 2.12. Homology modeling and molecular docking for determining the interface binding of the HuScFv to the NS1 protein

Details of the methods are given in Supplemental Materials and Methods 4.

### 2.13. ELISA inhibition for verification of the phage mimotopes

Phage clones displaying the mimotopes were propagated in ER2738 *E. coli* and the titers of the amplified phages were determined according to manufacturer's instruction (New England Biolabs, USA). Phage mimotope preparations (50 μl) containing various amounts of phages ($10^6$, $10^7$ and $10^8$ pfu) were mixed individually with fixed amount (5 μg) of HuScFv (50 μl) and incubated at 37 °C for 1 h. The HuScFv mixed with M13KO7 phage served as background binding control. F-NS1 coated wells added with the HuScFv served as 100% binding (maximum binding). After washing, rabbit anti-E tag antibody, goat anti-rabbit immunoglobulin-HRP conjugate and ABTS substrate were added, respectively. $OD_{405nm}$ of the content of each wells were determined. The % ELISA inhibition was calculated:

$$\%\ \text{ELISA inhibition} = [(OD_{405nm}\ \text{of maximum binding} - OD_{405nm}\ \text{of test}) \div (OD_{405nm}\ \text{maximum control})] \times 100$$

### 2.14. Effect of NS1 specific-HuScFv on virus infected cells

Expression levels (mRNA) of genes encoding IRF3 and IFN-β in influenza virus infected cells incubated with NS1 specific-HuScFv were compared with the mRNA in infected cells without the HuScFv treatment. A/duck/Thailand-Nong-Khai/KU-56/2007 (H5N1) virus mixed with HuScFv of clone no. 10, irrelevant HuScFv and antibody diluent containing homogenate of BL21(DE3) *E. coli* carrying empty pET32c$^+$ vector were added immediately after mixing to A549 cells in separate wells and kept for 2 h before the fluid in each well was replaced with fresh medium containing the respective HuScFv or controls and the cells were incubated further for 2 h. Total RNA was extracted from the infected cells of individual wells using RNeasy Mini kit (Qiagen) and quantified by using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Two pairs of primers specific to IRF3 and IFN-β coding gene sequences, i.e., *IRF3*: forward 5′-CTTGGAAGCACGGCCTAC-3′ and reverse 5′-CGGAAATTCCTCTTCCAGGT-3′ and *IFN-β*: 5′-GTCTCATTCCAGCCAGTGCT-3′ and reverse 5′-TGGCAATTGAATGGGAGGCT-3′, were designed from Genbank (accession no. Z56281 and NM_002176, respectively) by using Primer3 software. PCR master mix (12.5 μl) was prepared on ice with 2× Brilliant II SYBR® Green QRT-PCR Master Mix (Agilent), RT/RNase block enzyme, 200 nM of each primer and RNA solution (equal amount of total RNA). The real-time RT-PCR was carried out by using Mx3000P QPCR System machine (Agilent) at 42 °C for 1 h, initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. To analyze the dissociation curve, thermal profile of 95 °C for 1 min, then ramped down to 60 °C (0.5 °C/s) for 45 s and ramped up to 95 °C was used.

### 2.15. Statistical analysis

Differences among tests and controls were compared by using one way analysis of variance (ANOVA). Independent-*t*-test was used for analysis of the IRF3 and IFN-β mRNA expression levels. $p < 0.05$ was significantly different.

## 3. Results

### 3.1. Production of NS1 specific HuScFv and their characteristics

Recombinant F-NS1 and R and E domains of the NS1 were produce successfully. Supplemental Fig. 1A shows PCR amplicon of the DNA sequence coding for the F-NS1 (~675 bp) and the IPCR amplicons of the NS1 R and E domains with pET20b$^+$ vector (~3900 and ~3700 bp, respectively). Western blot patterns of the recombinant proteins in respective transformed *E. coli* lysates are illustrated in Supplemental Fig. 1B. The molecular masses of the recombinant F-NS1, R and E proteins were 30, 13 and 24 kDa, respectively. Deduced amino acid sequence (225 residues) of the recombinant F-NS1 is shown in Supplemental Fig. 1C.

Soluble recombinant F-NS1 protein was used as the antigen in the phage bio-panning for selecting NS1 bound phage clones. From the single round panning, 30 clones of the HB2151 *E. coli* grown on the selective agar plate were picked randomly and screened for the presence of the *huscfv* sequences (~1,000 bp); 25 clones (83%) were positive (Supplemental Fig. 2A). Among the 25 clones, 15 clones could express HuScFv (Supplemental Fig. 2B).

The DNA banding patterns (RFLP) of the *Mva*I cut-*huscfv* sequences of the 15 phagemid transformed HB2151 *E. coli* clones (clone nos. 1–15) that could express HuScFv are shown in Supplemental Fig. 3. There were 14 different banding patterns; two clones (nos. 3 and 8) had the same RFLP.

HuScFv prepared from the 15 *huscfv*-phagemid transformed HB2151 *E. coli* clones were tested by indirect ELISA against recombinant F-NS1 using BSA as antigen control and lysate of original HB2151 *E. coli* as negative antibody control. HuScFv of three clones (nos. 3, 10, and 11) gave significant ELISA signals above the controls (data not shown). HuScFv of these clones were tested for binding to the F-NS1 by dot ELISA. All clones were positive by the assay (Supplemental Fig. 4).

HuScFv prepared from the lysates of the *E. coli* clone no. 3 bound to SDS–PAGE separated-recombinant R domain of the NS1 protein while lysates of the *E. coli* clone nos. 10 and 11 bound to the NS1 E domain (Fig. 1).

### 3.2. NS1 specific-HuScFv inhibited influenza virus replication in the infected MDCK cells

$Log_{10}$ of plaque numbers of A/duck/Thailand/Nong-khai/KU56/2007 (H5N1) of tests a and b and control (c) are shown in Fig. 2. The plaque numbers of tests a and b (virus infected cells were grown in HuScFv-10 supplemented medium) were significantly less than the control (grown in HuScFv deprived-medium) throughout the course of the experiment (2–24 h). The plaque numbers during the early phase of the experiment (2–10 h) of the test a (virus mixed with HuScFv-10 before adding to MDCK cell monolayer) and test b (virus added directly into the cell monolayer) were not different statistically. Nevertheless during the late phase of the experiment (12–24 h), the test b had significantly higher plaque numbers than the test a ($p < 0.05$). Therefore, subsequent experiments for testing the effects of HuScFv of clone nos. 3, 10 and 11 on type A and type B virus were performed using the test a design.

Hemagglutination titers of the influenza A and B viruses and Sendai virus control in the culture supernatants of the MDCK cells collected at 24 h post-infection with the viruses that had been exposed to the NS1 specific-HuScFv of clone nos. 3, 10 and 11, irrelevant HuScFv and negative HuScFv controls are shown in Table 2.

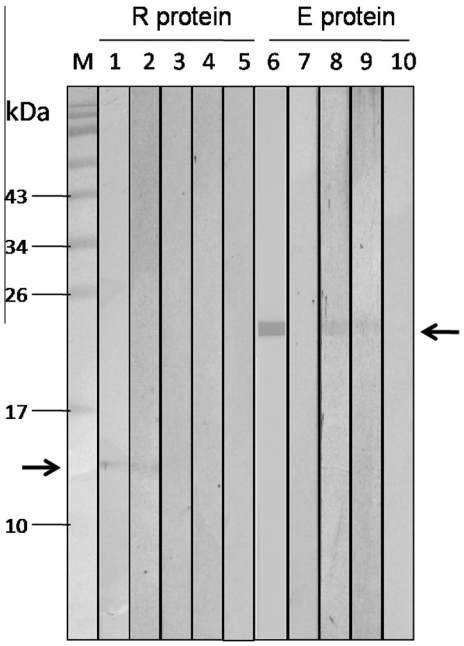


**Fig. 1.** Results of Western blot analysis for determining the NS1 domain bound by the HuScFv of clones no. 3, 10 and 11. (M) PAGE Ruler protein ladder. Lanes 1–5, SDS–PAGE separated-6x His tagged-recombinant R protein; lanes 6–10, SDS–PAGE separated-6x His tagged-E protein. Lanes 1 and 6 were probed with mouse monoclonal anti-6x His antibody (positive controls). Lanes 2 and 7, 3 and 8, and 4 and 9 were probed with HuScFv of clone nos. 3, 10 and 11, respectively. Lanes 5 and 10 were probed with irrelevant HuScFv control. The numbers at the left are protein molecular masses in kDa. The HuScFv of clone no. 3 bound only to the R protein at ~13 kDa (lane 2) (lower arrow) while HuScFv of clone nos. 10 and 11 bound to E domain at ~24 kDa (lanes 8 and 9, respectively) (upper arrow).

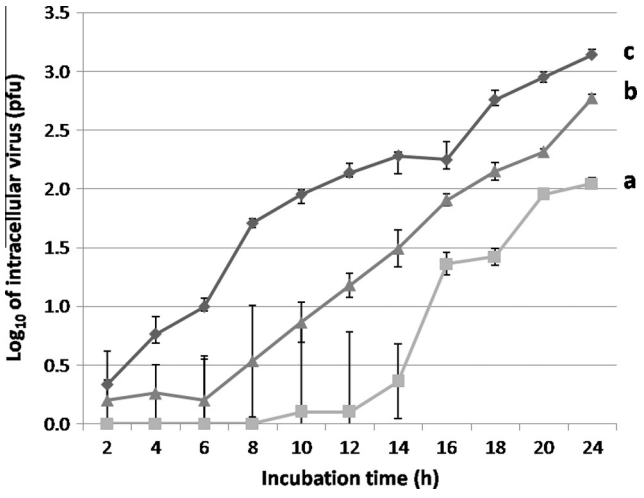


**Fig. 2.** Growth curves of influenza A/duck/Thailand/Nong-khai/KU56/2007 (H5N1). (A) Virus-HuScFv mixture was added to cells, 2 h adsorption, removed extracellular virus, washed, incubated in HuScFv containing medium; (B) virus was added directly to cells, 2 h adsorption and the infected cells were treated similarly to A; (C) virus was mixed with diluted lysate of original BL21 (DE3) *E. coli* before adding to the cells, 2 h adsorption, washed and the infected cells were grown in the medium containing diluted lysate of the *E. coli*. Plaque assay was performed for enumeration of the virus plaques in the infected cells of all treatment at every 2 h until 24 h (three wells/treatment/time point). The plaque forming units (pfu) of test a were less than tests b and c ($p < 0.05$ by ANOVA).

The HuScFv of all three clones could mediate a marked reduction of the amounts of the viruses released into the culture fluids by 2–64 fold when compared with the controls containing the virus infected cells that had been exposed to irrelevant HuScFv and lysate of BL21 (DE3) *E. coli* carrying pET32c$^+$ (negative HuScFv control). The HuScFv could not reduce the amounts of Sendai virus released into the culture fluids.

Fig. 3 shows cell ELISA results for determining the amounts of native NS1 and M2 proteins in MDCK cells infected with influenza viruses including A/duck/Thailand-Nong-Khai/KU-56/2007 (H5N1), A/dog/Thailand-Suphanburi/KU-08/2004 (H5N1), A/swine/Iowa-15/30 (H1N1), A/Thailand/CU-67/2006 (H1N1), A/CTB/32/11 (H1N1) which is A/California/7/2009 (H1N1)-like virus, A/swine/Thailand/KU-21/2004 (H3N2) and A/SRTN/23/11 (H3N2) which is A/Perth/16/2009-like virus that had been exposed to NS1 specific-HuScFv of clone nos. 3, 10 and 11, irrelevant HuScFv, negative HuScFv control. Control experiments using Sendai virus were included also as virus control. The $OD_{405nm}$ of the cells infected with influenza A viruses that had been exposed to the NS1 specific-HuScFv were much lower than the controls ($p < 0.01$) except for the HuScFv of clone no. 11 which the amount of intracellular NS1 of A/CTB/32/11 (H1N1) was not different statistically ($p > 0.05$) from the cells infected with the irrelevant HuScFv-treated virus although it was different statistically from the negative HuScFv control ($p < 0.05$). The HuScFv could not reduce the amounts of intracellular Sendai virus as detected by cell ELISA using chicken anti-Sendai virus polyclonal antibody.

Fig. 4 shows the results of the comparative real-time RT-PCR for comparing the number of HA and NA coding genes in MDCK cells infected with the B/TAK/42/11 (B/Brisbane/60/2008-like virus) that had been exposed to the HuScFv of clone nos. 3, 10 and 11, irrelevant HuScFv, and negative HuScFv control. Sendai virus exposed to the HuScFv served as control. The relative amounts of the HA and NA genes were lower ($p < 0.01$) in the cells infected with the influenza B virus that had been exposed to the NS1 specific-HuScFv of clone nos. 10 and 11 compared with irrelevant and negative HuScFv controls. The relative amounts of both gene sequences were not different between the virus treated with the HuScFv of clone no. 3 and the irrelevant HuScFv control ($p > 0.05$) but both were lower than those of the negative antibody control ($p < 0.01$). The HuScFv of all clones did not reduce the amounts of N protein mRNA of the Sendai virus compared to the controls.

The results of hemagglutination, cell ELISA and comparative real-time PCR indicated that the NS1 specific HuScFv of all three clones could interfere with replication of influenza viruses belonging to different subtypes and types including avian H5N1, swine H1N1 and H3N2, human H1N1 and H3N2 and influenza B virus. The low effectiveness of the HuScFv of clone no. 11 on replication inhibition of the A/CTB/32/11(H1N1) which is the A/California/7/2009(H1N1)-like virus might be due to less avidity of the binding of the HuScFv on the NS1 protein of this virus. Detail investigation is needed to elucidate this speculation. The irrelevant HuScFv specific to puffer tetrodotoxin also exerted inhibition of the influenza B virus replication as much as did by the HuScFv of clone no. 3 (Fig. 4). The irrelevant HuScFv might cross-react with some portion of the influenza B virus NS1 protein or other virus protein(s) that involved in the replication process and exerted the observed placebo effect.

### *3.3. Effects of HuScFv on native NS1 production in the influenza virus infected cells*

Fig. 5 illustrates the results of the confocal microscopy for detecting the amount of native NS1 in MDCK cells infected with A/duck/Thailand-Nong-Khai/KU-56/2007(H5N1) and A/swine/Iowa-15/30(H1N1) that had been exposed to the HuScFv of clone no. 10 (representative) grown in the HuScFv supplemented medium in comparison with the infected cells

**Table 2**
Reciprocal hemagglutination titers in the culture supernatants of MDCK cells infected with different influenza viruses and Sendai virus that had been exposed to HuScFv of clone nos. 3, 10 and 11.

| Name of virus isolate (subtype) | Reciprocal hemagglutination titer of the virus in the cell culture supernatant | | | | |
|---|---|---|---|---|---|
| | Negative HuScFv control[a] | Irrelavant HuScFv[b] | HuScFv of clone no. | | |
| | | | 3 | 10 | 11 |
| A/duck/Thailand-Nong-Khai/KU-56/2007 (H5N1) | 128 | 128 | 2 | 32 | 8 |
| A/dog/Thailand-Suphanburi/KU-08/2004 (H5N1) | 128 | 128 | <2 | <2 | <2 |
| A/swine/IA-15/30 (H1N1) | 32 | 32 | 16 | 4 | 4 |
| A/Thailand/CU-67(2006) (H1N1) | 8 | 4 | <2 | 2 | <2 |
| A/CTB/32/11 (A/California/7/2009(H1N1)-like virus) | 8 | 8 | <2 | <2 | neg |
| A/swine/Thailand/KU-21/2004 (H3N2) | 4 | 4 | <2 | <2 | <2 |
| A/SRTN/23/11 (A/Perth/16/2009(H3N2)-like virus) | 4 | 4 | 4 | <2 | <2 |
| B/TAK/42/11 (B/Brisbane/60/2008-like virus) | 8 | 4 | 2 | neg | <2 |
| Sendai virus, ATCC strain 52 (Fushimi) | 8 | 8 | 8 | 8 | 8 |

[a] Virus mixed with lysate of HB2151 *E. coli* before adding the MDCK cells.
[b] Virus mixed with irrelevant HuScFv (specific to puffer tetrodotoxin) before adding to MDCK cells; neg, negative result or no visible agglutination of the red blood cells; <2, there were some agglutination of erythrocytes but the agglutination was not complete.

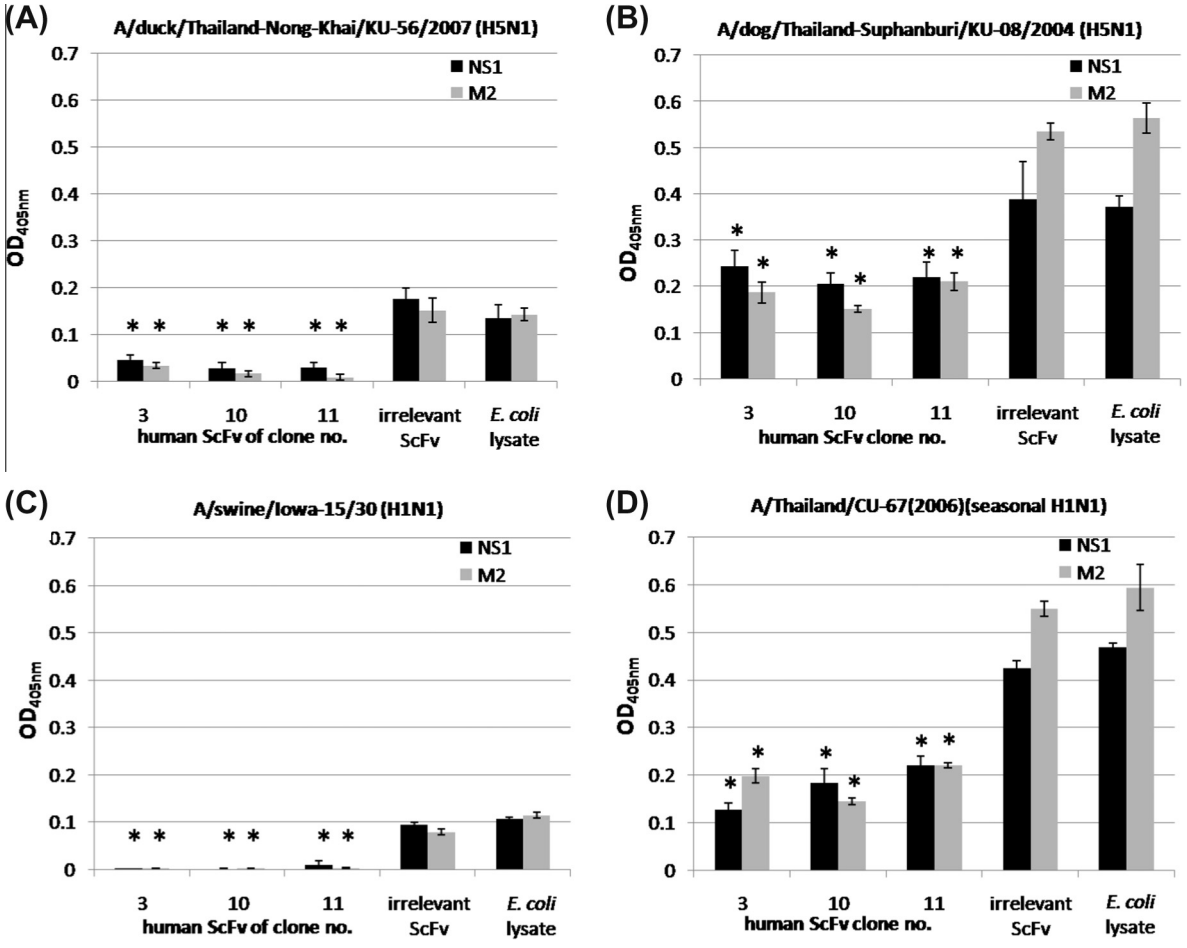


**Fig. 3.** Results of cell-ELISA for determining the amounts of NS1 and M2 proteins of influenza A viruses and Sendai virus in MDCK cells infected with the viruses that had been exposed to NS1 specific-HuScFv of clone nos. 3, 10 and 11, irrelevant HuScFv and negative HuScFv control. (A–H), cells infected with A/duck/Thailand-Nong-Khai/KU-56/2007 (H5N1), A/dog/Thailand-Suphanburi/KU-08/2004 (H5N1), A/swine/Iowa-15/30 (H1N1), A/Thailand/CU-67/2006 (H1N1), A/CTB/32/11 (H1N1) which is A/California/7/2009 (H1N1)-like virus, A/swine/Thailand/KU-21/2004 (H3N2), A/SRTN/23/11 (H3N2) which is A/Perth/16/2009 (H1N1)-like virus and Sendai virus (ATCC strain 52, Fushimi), respectively. ∗, significantly different from negative ScFv and irrelevant HuScFv controls at $p < 0.01$. #, different from negative HuScFv control ($p < 0.01$) but not different from irrelevant HuScFv ($p > 0.05$).

exposed to lysate of pET32c$^+$ transformed BL21 (DE3) *E. coli* (negative HuScFv control) grown in HuScFv deprived medium. Much less NS1 protein (green fluorescence) could be detected in the cells exposed to the NS1-specific HuScFv than in the control cells.

### *3.4. NS1 specific-HuScFv, native NS1 and lipid raft in the influenza virus infected cells*

Results of the sectional laser confocal microscopic experiments for locating the NS1 specific-HuScFv (green), native NS1 (red) and

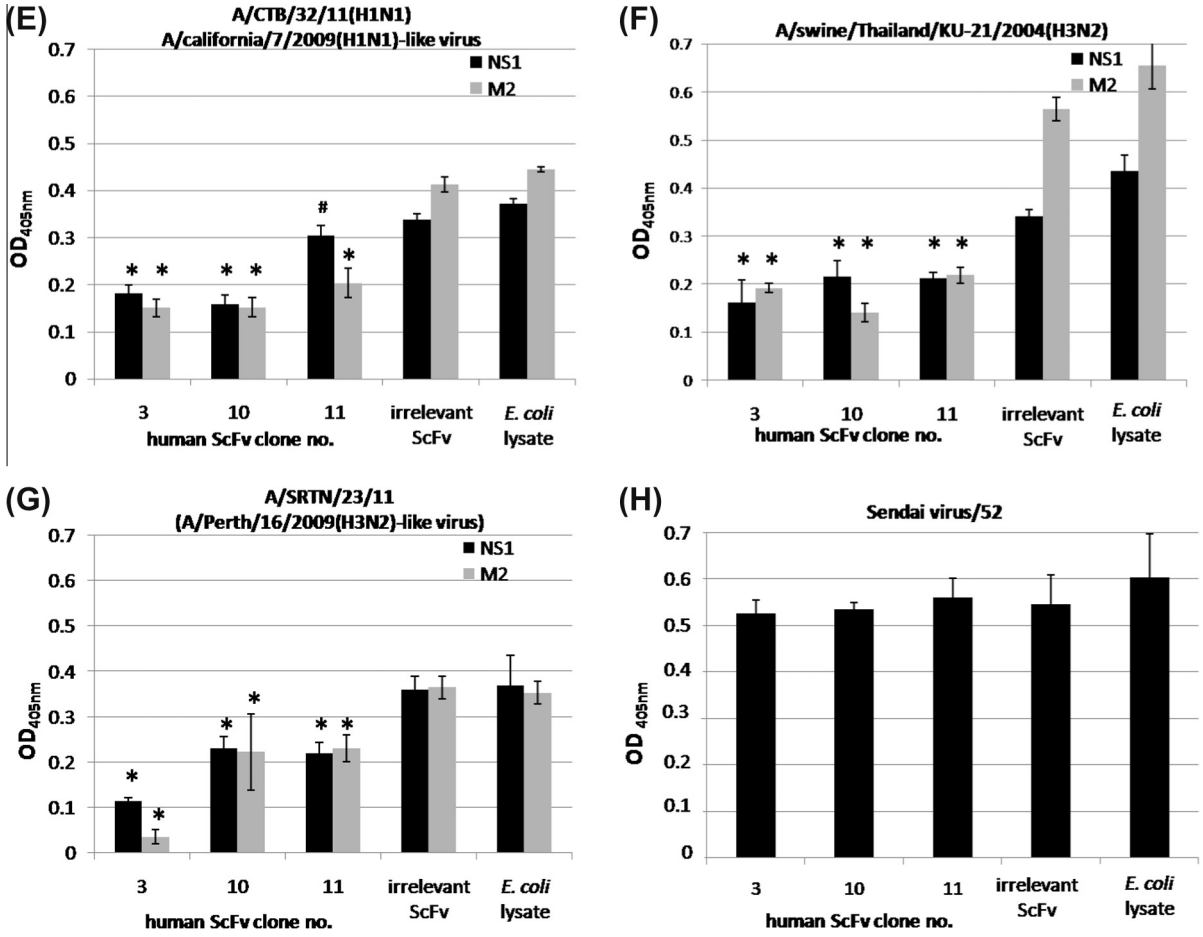


**Fig. 3** (*continued*)

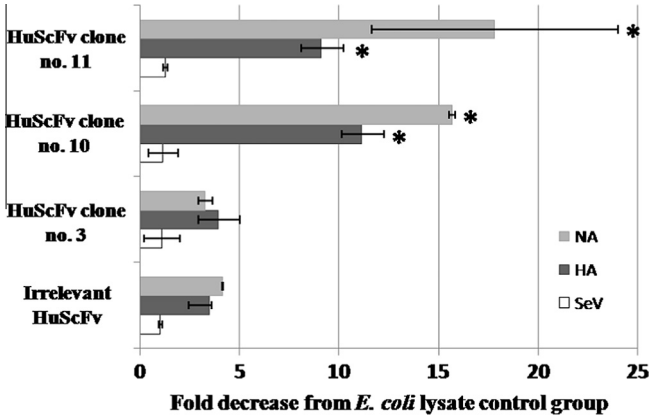


**Fig. 4.** The results of comparative real-time PCR showing fold decrease of mRNAs coding for HA and NA of influenza B virus (B/TAK/42/11 and N protein of Sendai virus (SeV) in MDCK cells infected with the viruses and that had been exposed to the HuScFv of clone nos. 3, 10 and 11 and irrelevant HuScFv compared with negative antibody (*E. coli* lysate) control. ∗, different from HuScFv clone no. 3 and irrelevant HuScFv ($p < 0.05$).

lipid raft (blue) in MDCK cells infected with A/duck/Thailand-Nong-Khai/KU-56/2007(H5N1) that had been exposed to the HuScFv of clone no. 10 and grown in the HuScFv-supplemented medium for 4 h are shown in Fig. 6. The HuScFv could be seen intracellularly at different 2.4 μm sectional layers of the cells indicating that the small antibody fragments could enter the influenza virus infected cells.

### 3.5. Phage mimotopes and NS1 epitopes that bound to the HuScFv

The 12 amino acid sequences deduced from the genomes of the respective peptide display phage clones that bound to the HuScFv were aligned with the NS1 amino acid sequence of the F-NS1 of the A/duck/Thailand/144/2005 in order to locate the residues of the NS1 protein that could interact with the HuScFv. The Kalign program predicted that the phage mimotopes derived from panning with the HuScFv of clone no. 3 bound to residues 7–18 in the RNA binding (R) domain of the NS1 protein (Fig. 7A). The amino acid residues 75–90 and 179–190 of the E domain were predicted as the binding sites of the HuScFv of clone nos. 10 and 11, respectively (Fig. 7B and C).

### 3.6. Interface binding of the HuScFv to the NS1 protein

The structures with the highest bit scores were used as templates for homology modeling. For the NS1 template, the crystal structure of the influenza A, NS1A, protein which formed complex with the F2F3 fragments of the human cellular factor, CPSF30 (PDB ID: 2RHK) was used as the template. PDB ID: 2GHW and 1NQB were used as the templates of HuScFv of clone nos. 3 and 10 and clone no. 11, respectively. All modeled structures were subjected to the Ramachandran plot assessments. The results of the plots indicated that all structures were appropriate for use in the molecular docking analysis (data not shown).

The docked poses of the modeled NS1 and HuScFv were demonstrated in the forms of the binding residues and the binding energy. The predicted interactive residues between the NS1 and the

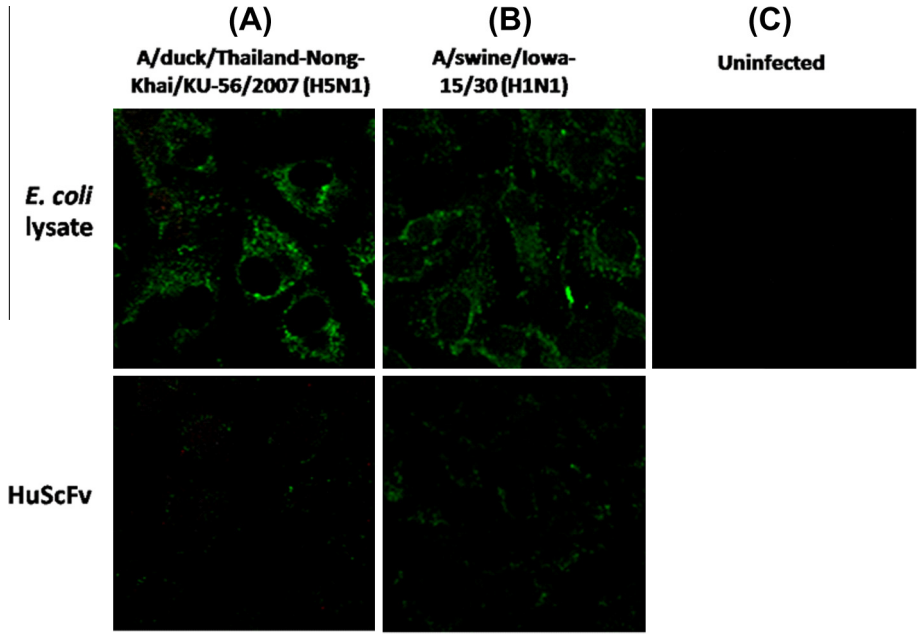


**Fig. 5.** Results of confocal microscopy for detecting native NS1 in MDCK cells infected with influenza viruses that had been exposed to NS1 specific-HuScFv compared with the infected cells exposed to negative HuScFv control. (A) MDCK cells infected with A/duck/Thailand-Nong-Khai/KU-56/2007(H5N1). (B) MDCK cells infected with A/swine/Iowa-15/30(H1N1). (C) Uninfected MDCK cells. NS1 (green fluorescence) are abundant in the control cells that were infected with the viruses exposed to negative HuScFv control and grown in the medium containing diluted lysate of *E. coli* that could not produce HuScFv (upper panels of A and B). The fluoresced-NS1 amounts were much reduced in the cells infected with the same viruses that had been exposed to the NS1 specific-HuScFv of clone no. 10 (representative) and grown in the HuScFv containing medium (lower panels of A and B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HuScFv revealed by the computerized molecular docking are indicated by black shades in Fig. 8. The NS1 amino acids were residues 1–190 while the amino acids of HuScFv of clone nos. 3, 10 and 11 were residues 191–441, 191–439 and 191–439, respectively. The interactive amino acid residues were relatively conformed to the results of phage mimotope/epitope identification and Western blot analysis. The binding energy of the complexes between the NS1 protein and the HuScFv of clone nos. 3, 10, and 11 were −9.95, −22.3, and −20.5 kcal/mol, respectively, implying that the interactions between the ligand and the antibodies occurred spontaneously which is the nature of the antigen–antibody reaction. Supplemental Fig. 5 shows hypothetical models of the binding sites of the HuScFv (ribbons) of clone nos. 3 (yellow), 10 (pink) and 11 (blue) on the NS1 molecular surface (green surface model).

### 3.7. Inhibition of HuScFv binding to F-NS1 by phage mimotopes

Results of the ELISA inhibition for determining the ability of phage mimotopes in inhibiting the HuScFv binding to the F-NS1 are shown in Supplemental Fig. 6. Binding of the HuScFv to the F-NS1 was inhibited by the HuScFv-phage mimotopes in a dose dependent manner implying that the NS1 peptides matched with the phage mimotope sequences were the HuScFv binding sites which validated the mimotope search and epitope mapping results.

### 3.8. NS1-specific HuScFv enhanced expression of IFN-β and IRF3 genes in virus infected cells

Results of comparative real-time RT-PCR for determination of expressions of mRNAs of IRF3 and IFN-β coding genes in A549 cells infected with A/duck/Thailand-Nong-Khai/KU-56/2007 that had been exposed to HuScFv of clone no. 10, irrelevant HuScFv and negative HuScFv control are shown in Fig. 9. The fold increase in IRF3 and IFN-β mRNAs of the virus infected cells treated with the NS1 specific HuScFv were higher than treated with BL21 (DE3) lysate control (14.75 and 4.95 fold, respectively) ($p < 0.05$). The increase in IRF3 and IFN-β mRNAs of the virus infected cells treated with HuScFv and the irrelevant HuScFv were different significantly ($p < 0.05$) although the irrelevant HuScFv showed some non-specific background. The results indicated that the NS1 specific-HuScFv could rescue the infected cells from the NS1 and restored the cellular innate response.

## 4. Discussion

In this study, soluble recombinant full length NS1 (F-NS1) of A/duck/Thailand/2004 (H5N1) was produced. The protein had the relative molecular mass of ~30 and contained 225 amino acids which conformed to the data reported previously (Suwannakhon et al., 2008). The recombinant F-NS1 lacked part of the second NLS2 (located between amino acids 223 and 237) and the whole nucleolar localization signal (NoLS) which are basic amino acid residues at positions 231 and 232 (Suwannakhon et al., 2008). Nevertheless, the protein retained other features of the highly conserved NS1 of the avian H5N1 influenza viruses (allele A) including a stretch of basic amino acids DRLRR at positions 34–38, amino acid deletions at 80–84 and amino acid change at residue 92 (D92E) (Suwannakhon et al., 2008). Because of the residue deletions, the E-domain hydrophobic pocket of the NS1 known to be the site for binding of the 30-kDa subunit of the cleavage and polyadenylation specific factor which is important for the 3′ end pre-mRNA processing (Nemeroff et al., 1998; Twu et al., 2007; Xia et al., 2009) of the F-NS1 is formed by amino acids at different positions from the human influenza A viruses.

The recombinant F-NS1 was used as antigen in a single round phage panning for selecting phage clones that bound to the protein from a previously constructed human ScFv phage display library. This phage library had been used successfully for selection and production of HuScFv specific to a variety of molecules (Kulkeaw

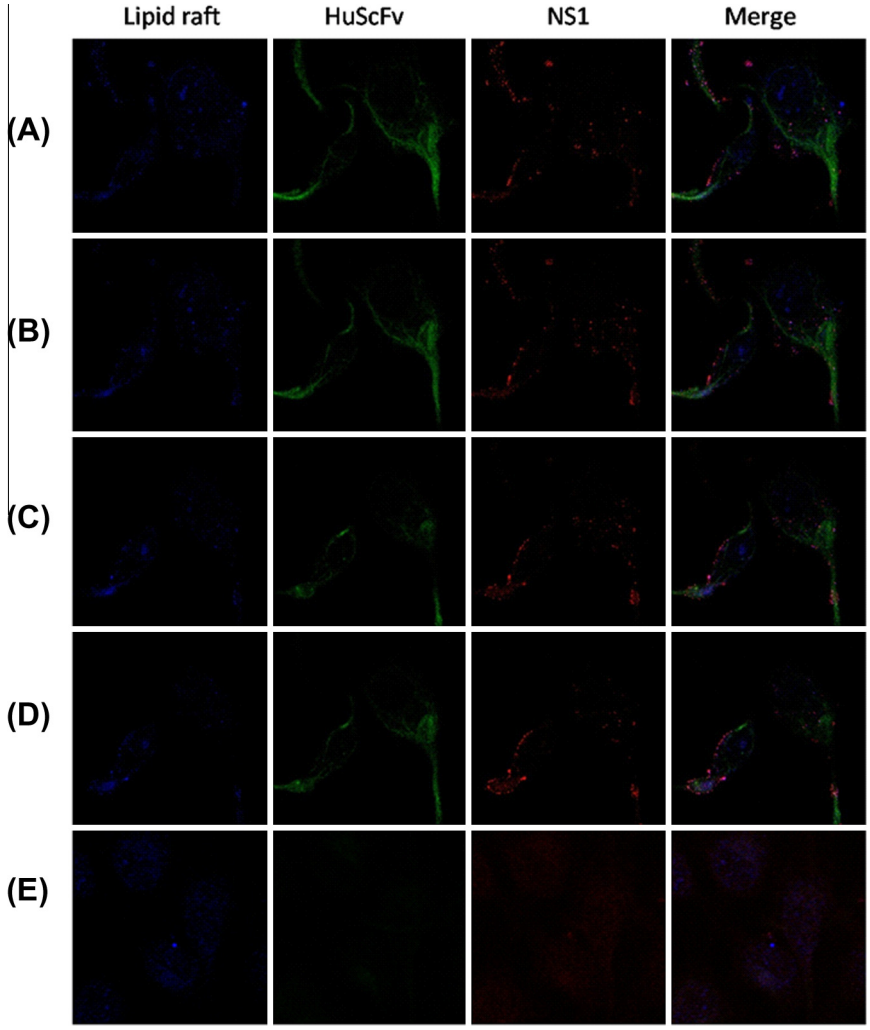


**Fig. 6.** Sectional laser confocal microscopy of A/duck/Thailand-Nong-Khai/KU-56/2007(H5N1) infected MDCK cells incubated with HuScFv specific to NS1 (clone no. 10) or negative antibody control. (A–D) Four serial 2.4 μm sections showing (from left to right) lipid raft (blue), HuScFv (green), NS1 (red) and merged in the infected cells, respectively. (E) Non-infected cells incubated with HuScFv of clone no. 10. The results indicate that the HuScFv could enter the virus infected cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2009; Maneewatch et al., 2009; Indrawattana et al., 2010; Poungpair et al., 2010; Chulanetra et al., 2012). The single round phage panning was performed as it is a time saving and economic procedure compared with the multiple round panning (Thanongsaksrikul and Chaicumpa, 2011; Winter et al., 1994). The repeated panning may enrich the antigen specific phage clones but the *huscfv* genes may be lost from the phagemids in the successive panning rounds rendering a selection of falsely positive phage clones (personal observation). The single round procedure was used effectively for the production of HuScFv specific to many other influenza virus proteins including polymerases (Thathaisong et al., 2008), H5 (Maneewatch et al., 2009) and M1 (Poungpair et al., 2009, 2010) as well as other molecules such as snake venom (Kulkeaw et al., 2009; Chavanayarn et al., 2012), tetanus and botulinum neurotoxins (Indrawattana et al., 2010; Thanongsaksrikul et al., 2010), tetrodotoxin (Chulanetra et al., 2012) and hepatitis C virus RNA dependent RNA polymerase (Thueng-in et al., 2012). By using the F-NS1 as the panning antigen, phage clones displaying HuScFv that bound specifically to both the RNA binding (R) and the effector (E) domains could be selected from the one time panning. The *huscfv* sequences coding for the HuScFv of the three clones had different DNA restriction patterns (RFLP) and the deduced amino acids in their CDRs were also different (data not shown), indicating primarily that the HuScFv of these clones should have different epitope specificities. The speculation was partly confirmed by the results of the Western blot analysis which revealed that the HuScFv of the clone no. 3 bound to the R domain and those of the clone nos. 10 and 11 bound to the E domain of the NS1 protein.

Influenza viruses produce large amount of the NS1 protein in the infected host cells. The protein can be found in both nucleus and cytoplasm (Greenspan et al., 1988). In the nucleus, the protein has been shown to complex with the cellular CPSF30 and/or PABPII proteins and inhibited the 3′ end processing of the pre-mRNA resulting in inhibition of the host protein synthesis and enhancement of the viral replication (Noah et al., 2003). In cytoplasm, NS1 binds to dsRNA and deprived this virus component from host OAS, RIG-1 and PKR which consequently limits the host anti-viral immunity. Confocal microscopy revealed that the amounts of the native NS1 in the MDCK cells infected with the HuScFv exposed-influenza viruses were reduced markedly. Thus, the reduction of the virus replication might be due to the HuScFv-mediated interference of the intracellular NS1 production and functions which consequently suppressed the virus replication and rescued the host innate immune apparatus. The different inhibition efficiency of HuScFv to various strains of viruses might be due to different alleles of the NS1 between avian and mammalian viruses. The HuScFv were derived from phage bio-panning with NS1 allele A of avian H5N1 and they were highly efficient against H5N1 viruses

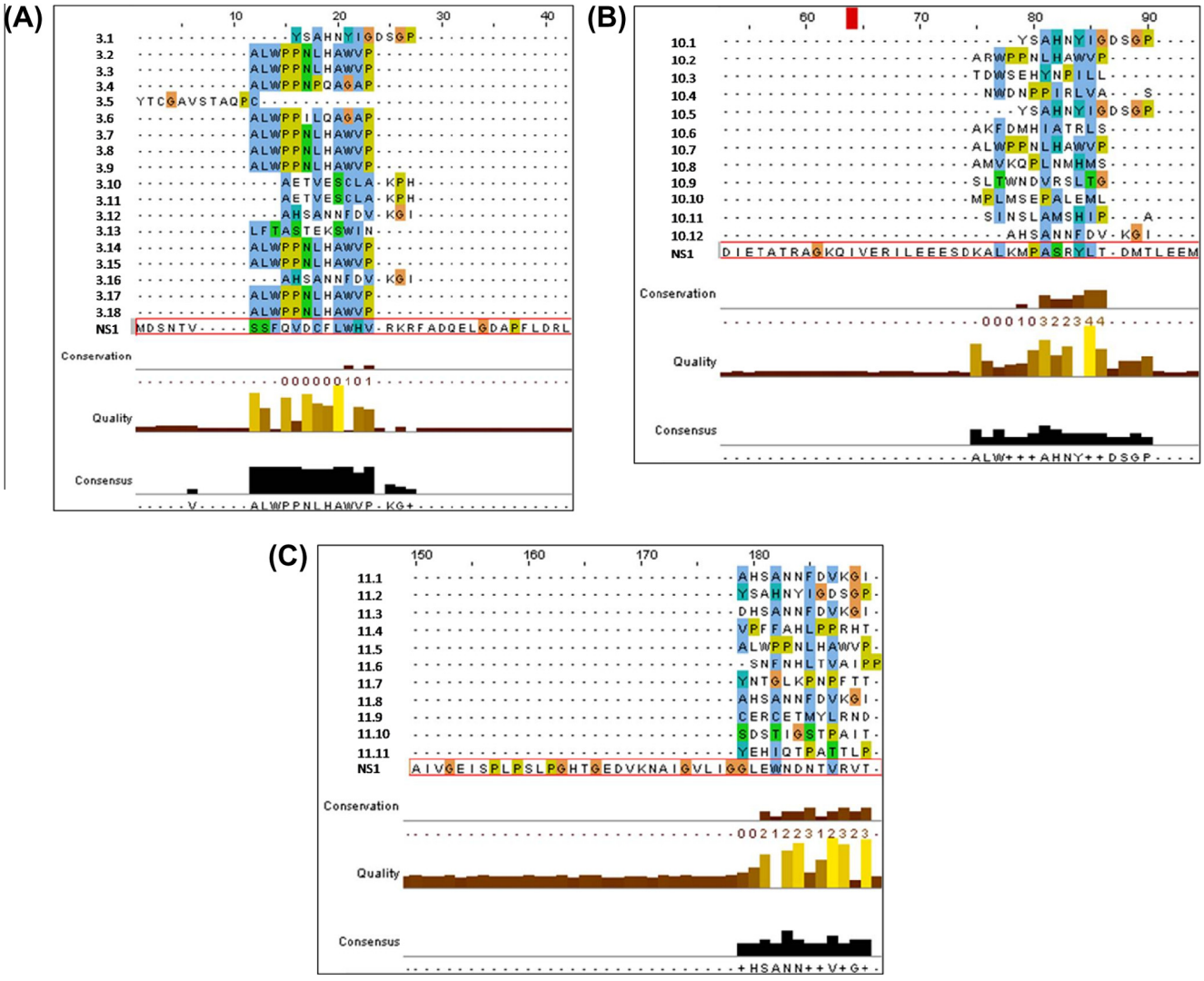


**Fig. 7.** Multiple alignments of the HuScFv bound-phage mimotopes with the NS1 sequence by using Kalign program for predicting the NS1 epitopes. (A) Residues 7–18 in the RNA binding domain of the NS1 protein were predicted as the binding site of HuScFv of clone no. 3. (B) and (C) Residues 75–90 and 179–190 of the E domain were predicted as binding sites of the HuScFv of clone nos. 10 and 11.

(homologous subtype) and less efficient to the heterologous viruses. Nevertheless, significant inhibition was observed for all influenza viruses tested.

It was found that the HuScFv mediated virus replication inhibition of the test b (virus was added directly to the cells to allow entry before culturing the infected cells in HuScFv supplemented medium) was not as good as the experiments which the HuScFv was mixed with the virus and added immediately to cells (test a). This might be because some of the early entered virus of the test b produced NS1 and the NS1 could enhance the virus replication during the first 2 h before the HuScFv could enter the cells and interfered with the NS1 activities.

Usually, normal mammalian cell membranes are formidable for intact antibody molecules as well as the single chain antibody variable fragments (ScFv, mol. wt. 25–35 kDa) which are ~5 times smaller than the intact IgG. Thus, antibodies are inaccessible to the intracellular targets. In this study, however, the HuScFv specific to the NS1 protein was found inside the influenza virus infected MDCK cells. It has been known that during several virus infections, e.g., HIV-1, Dengue virus, influenza virus, plasma membranes of the infected cells have increased permeability especially at the lipid raft areas where the budding of the virus progeny is taking place (Cloyd and Lynn, 1991; Frolov et al., 2003; Talavera et al., 2004). This feature together with the presence of the intracellular target is likely to facilitate and attract the cellular entry of the HuScFv.

The ELISA inhibition results demonstrated the ability of the phage mimotopes in blocking the binding of the respective HuScFv to the F-NS1 which implied that the phage peptides and the NS1 residues which were epitopes of the HuScFv were analogous. The finding that the mimotope sequence of the HuScFv of the clone no. 3 matched with the residues 7–18 in the RNA binding domain of the NS1, not only conformed to the Western blot result, but also indicated that the HuScFv should be able to bind to the same region on the native NS1 in the virus infected cells. The R38 and K41 of the functional NS1 molecule are known to be critical for binding of the R domain to the virus dsRNA and depriving the target from the OAS, RIG-1 and PKR (Guo et al., 2007; Min et al., 2007; Min and Krug, 2006). Although the HuScFv of the clone no. 3 did not interact directly with the two amino acids, nevertheless, binding to the residues 7–18 might cause steric hindrance of the dsRNA binding by the R38 and K41 which in the effect might interfere with the NS1 mediated suppression of the interferon based-immunity of the host. The residues 81–113 served as the NS1 binding site to the eIF4GI which causes an enhancement of the viral mRNA translation (Lin et al., 2007). Binding of the HuScFv of clone no. 10

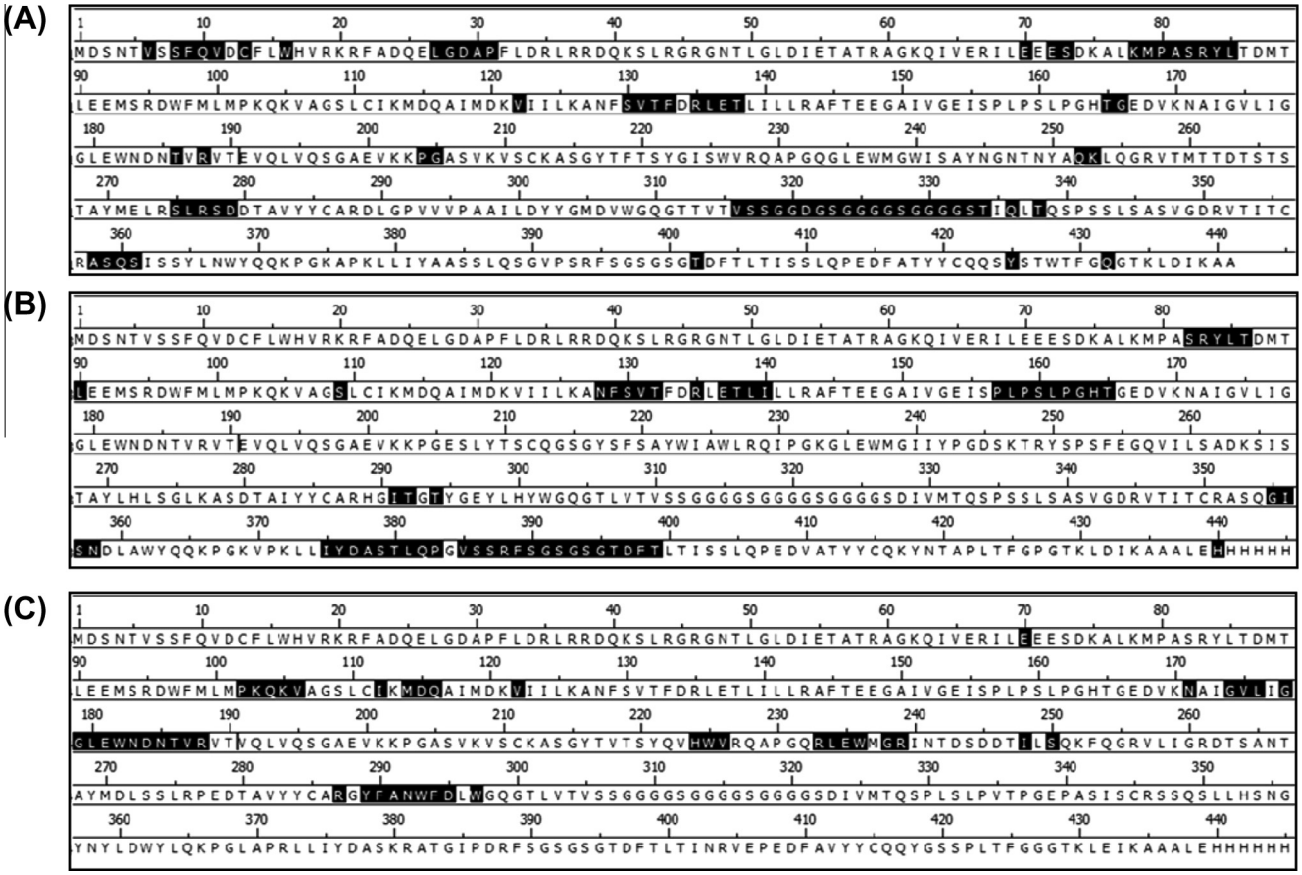


**Fig. 8.** Predicted NS1 residues bound by specific HuScFv. (A) HuScFv of clone no. 3 (residues 191–441), (B) HuScFv of clone no. 10 (residues 191–439) and (C) HuScFv of clone no. 11 (residues 191–439) which bound with the NS1 protein (residues 1–190). Black shades indicate interactive residues.

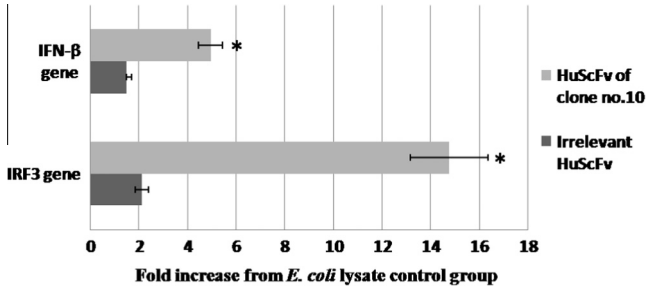


**Fig. 9.** Results of comparative real-time RT-PCR for detecting the levels of IRF3 and IFN-β mRNAs in the A549 cells infected with influenza virus A/duck/Thailand-Nong-Khai/KU-56/2007 that had been exposed to HuScFv of clone no. 10 or irrelevant HuScFv compared with the negative HuScFv (*E. coli* lysate) control. ∗, different from infected cells treated with irrelevant HuScFv ($p < 0.05$).

to the residues 75–90 of the NS1 E domain should interfere with the NS1 binding to the eIF4GI which the inhibition of viral replication was a consequence. The amino acids F103, M106, L144 and residues 184–188 of NS1 of human influenza viruses, which are F98, M101, L139 and 179–183 of the F-NS1 of this study, bound to the CPSF30 which is important for the 3′ end pre-mRNA processing. The HuScfv of clone no. 11 interacted with residues 179–190 of the NS1 protein. Thus the mechanism which the HuScFv of clone no. 11 inhibited the virus replication should be due to the inability of the HuScFv-bound NS1 to intervene the host pre-mRNA maturation. The finding that the HuScFv did not reduce the Sendai virus replication indicated that the activity of the HuScFv are influenza virus-specific.

Influenza virus infected A549 cells treated with HuScFv had significant up-regulation of genes involved in the host innate immune response, i.e., IRF3 and IFN-β. The HuScFv alone did not have interferon agonistic activity on the mammalian cells (data not shown). Data indicated that the influenza virus NS1 specific-HuScFv, through NS1 function interference, could lead not only to the virus replication inhibition, but also restoration of the host innate immune response.

In conclusion, three different *huscfv*-phagemid transformed *E. coli* clones were selected from a human ScFv phage display library by means of a single round phage panning using recombinant full length NS1 protein of influenza A virus as the panning antigen. HuScFv of one *E. coli* clone bound to the NS1 R domain important for virus dsRNA binding while HuScFv of the other two clones bound to E domain at the NS1 sites necessary for host eIF4GI and CPSF30 interactions. The HuScFv could enter the influenza virus infected cells and interfered with the NS1 activities leading to restoration of expressions of the host genes involved in innate immune response and replication inhibition of influenza A viruses belonging to heterologous subtypes as well as the type B virus. The fully human single chain antibodies have high potential for developing further as a safe adjunctive therapeutic agent for mitigating symptom severity of influenza.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.07.019.

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