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# H5N1 NS genomic segment distinctly governs the influenza virus infectivity and cytokine induction in monocytic cells

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## Abstract

**Background:** The level of virulence of H5N1 highly pathogenic avian influenza (HPAI) virus was higher than those of the other virus subtypes. It has been suggested that the nonstructural (*NS*) gene might be a factor contributing to H5N1 HPAI virulence.

**Objectives:** To determine the efficiency of the NS genomic segment of H5N1 HPAI virus on governing viral infectivity and cytokine induction in monocytic cells compared to other virus strain/subtypes.

**Methods:** By reverse genetics, five reassortant influenza viruses carrying the NS genomic segment derived from seasonal influenza A(H1N1), 2009 pandemic A(H1N1), A(H3N2) or H5N1 HPAI virus in the backbone of A/Puerto Rico/8/34 H1N1 (PR8) virus were constructed together with the reassorted PR8 virus control, i.e., rgH1N1sea-NS, rgH1N1pdm-NS, rgH3N2-NS, rgH5N1-NS and rgPR8 viruses, respectively. These reverse genetics-derived viruses (rg-viruses) were used to infect monocytic cells for 24 hours prior to determining intracellular influenza nucleoprotein (NP) levels and cytokine induction by flow cytometry.

**Results:** U937 cells were significantly more susceptible to rgPR8 control virus than THP-1 cells; thus, U937 cells were chosen for further study. The number of U937-infected cells ($NP^+$ cells) and the numbers of infected cells that expressed IFN-α ($NP^+IFN\text{-}\alpha^+$ cell) obtained with rgH5N1-NS virus infection were significantly higher than the others, except for cells infected with the rgH1N1pdm-NS virus. Nevertheless, the numbers of U937 cells that expressed $NP^+IL\text{-}1\beta^+$ were comparable upon infection with any of the rg-viruses; almost none expressed TNF-α.

**Conclusions:** The H5N1 NS genomic segment distinctly up-regulated the viral infectivity and induction of IFN-α compared to the rgPR8, rgH1N1sea-NS and rgH3N2-NS viruses.

**Keywords:** Influenza nonstructural genomic segment, virus infectivity, cytokines, monocytic cells, reverse genetics, flow cytometry

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## Introduction

At present, influenza type A viruses are divided into 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes.[1,2] The virus subtypes A(H1N1) and A(H3N2) together with influenza B viruses are responsible for human infections worldwide.[2] The HA subtypes H1-H16 and NA subtypes N1-N9 are present in avian species,[2] while H17N10 and H18N11 are found in bats.[1] Influenza in natural avian hosts is usually mild or asymptomatic, but the highly pathogenic avian influenza (HPAI) viruses cause massive death in poultry population.[3] The H5N1 HPAI virus was the first avian influenza virus that transmitted directly from poultry to humans, according to a report from Hong Kong in 1997; this resulted in 18 infected patients with six deaths (fatality rate of 33.3%).[4] The H5N1

HPAI virus with higher virulence re-emerged in 2003 and continuously spread across countries and continents until the present day. As of February 2017, the re-emerged virus has infected humans in 16 countries, and the World Health Organization[5] reported an accumulated number of 856 laboratory-confirmed cases with 452 deaths (fatality rate of 52.8%). Accordingly, the H5N1 HPAI virus is the most virulent influenza virus, compared to the 31% fatality rate of the H7N9 avian virus and less than 0.5% for human viruses.[5] Patients infected with H5N1 HPAI virus mostly develop severe pneumonia with acute respiratory distress syndrome and multi-organ failure in which hypercytokine production or "cytokine storm" is the hallmark of the disease.[6,7]

The virulence of the H5N1 HPAI virus may be mediated by multiple factors. The amino acid substitution E627K in the *PB2* gene has been linked to higher virulence in mice.[8] Multiple basic amino acids at a cleavage site in the *HA* gene,[8] together with the resistance of HA protein to serum innate inhibitor,[9] provide the H5N1 HPAI virus with the ability to disseminate beyond the respiratory tract. Some evidence also suggests that the nonstructural (NS) protein may also play a role in the virulence of the H5N1 HPAI virus.[10-16]

Influenza A *NS* genes are more conserved than *HA* and *NA* genes. Nevertheless, the evolutionary tree of a large-scale analysis on 7,581 influenza NS nucleotide sequences revealed two distinct gene pools, i.e. NS alleles A and B. Allele A was further divided into eight lineages, I-VIII, and allele B was divided into two lineages, IX and X.[17] The NS genomic segment encodes for two proteins, NS1 and NS2 or nuclear export protein.[18] NS1 may possess several functions, in particular cytokine dysregulation, a factor responsible for severe influenza pathology.[10-12,19] In monocyte derived-macrophages (MDMs), H5N1 HPAI virus infection induces a higher level of cytokine production than that observed with the 2009 pandemic influenza A(H1N1) virus (H1N1pdm virus), and is sequentially followed by seasonal influenza virus subtypes H1N1 (H1N1sea virus) and H3N2.[20] Moreover, it has been shown that MDMs infected with the H1N1pdm virus produce higher level of cytokines than those infected with the A/Puerto Rico/8/1934 (H1N1) virus (PR8 virus).[21] This report was based on information obtained with wild type viruses; but the mechanism by which the gene might influence cytokine production was not further explored. Several reports have dissected the influence of the NS segment on cytokine induction by reverse genetics technology, but data across virus subtypes are still limited.[13-15,19,22]

In this study, NS genomic segment of the H5N1 HPAI virus was compared with those of the other virus subtypes on its efficiency to enhance viral infectivity and cytokine induction. Five kinds of (7+1) reverse genetic derived viruses (rg-viruses) carrying the NS genomic segment from the H5N1 HPAI, H1N1pdm, H1N1sea or H3N2 virus in the backbone of the PR8 virus together with the reassorted PR8 virus control were constructed. U937 and THP-1 monocytic cells were assessed for their susceptibility to reassorted PR8 virus infection by measuring the number of cells that expressed influenza nucleoprotein ($NP^+$ cells). Thereafter, numbers of U937 cells infected with the rg-viruses and expressed interferon-α ($NP^+IFN\text{-}\alpha^+$), tumor necrosis factor-α ($NP^+TNF\text{-}\alpha^+$ cells) or interleukin-1β ($NP^+IL\text{-}1\beta^+$ cells) were analyzed by flow cytometry.

## Methods

### *Cell lines*

MDCK (Madin-Darby canine kidney) cells were grown in Earle's minimum essential medium (EMEM) (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics. Human embryonic kidney (HEK)-293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS and antibiotics. U937 and THP-1 human monocytic cell lines were cultured in Roswell Park Memorial Institute medium 1640 (RPMI1640) (Gibco) supplemented with 10% FBS and antibiotics. U937 and THP-1 monocytic cell lines differ with respect to their origins and maturation stages.[23,24] THP-1 cells were derived from peripheral blood,[23] while U937 cells were derived from a pleural effusion.[24] THP-1 cells are monoblasts[23] but U937 cells are promonocytes,[24] suggesting that U937 are more mature than THP-1 cells.

### *Viruses*

The four influenza strains used in this study were A/Thailand/Siriraj-02/2006 (H1N1) seasonal virus (H1N1sea virus), A/Thailand/104/2009 (H1N1) pandemic virus (H1N1 pdm virus), A/Prachuap Khiri Khan/137/2013 (H3N2) virus, and highly pathogenic A/Thailand/1 (KAN-1A)/2004 (H5N1) virus. These viruses were propagated in MDCK cells maintained in virus growth media (VGM) containing EMEM supplemented with antibiotics. The VGM for these viruses, except H5N1 virus, was supplemented with 2 μg/ml of tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (TPCK-trypsin) (Sigma-Aldrich, St. Louis, MO).

### *Nucleotide sequencing*

The NS genomic segments of the study viruses were amplified by polymerase chain reaction for direct sequencing of the DNA products. Nucleotide sequences were deposited in the GenBank database under accession numbers KX721052, GQ229379, KX885853, and KX721053 for the NS segments of A/Thailand/Siriraj-02/2006 (H1N1), A/Thailand/104/2009 (H1N1), A/Prachuap Khiri Khan/137/2013 (H3N2), and A/Thailand/1 (KAN-1A)/2004 (H5N1), respectively.

### *Construction of reverse genetics-derived viruses*

Eight recombinant pHW2000 plasmids carrying each genomic segment of PR8 virus for construction of reassortant viruses was kindly provided by Prof. Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN, USA. Using the reverse genetics technique as described previously,[25] five kinds of reverse genetics-derived viruses harboring the NS genomic segment of H1N1sea, H1N1pdm, H3N2 or H5N1 virus in the backbone of the PR8 virus (7+1 rg-viruses) and the reassorted PR8 virus control were generated, and named rgH1N1sea-NS, rgH1N1pdm-NS, rgH3N2-NS, rgH5N1-NS and rgPR8 viruses, respectively. Briefly, NS genomic segments of the donor viruses were amplified using universal primers designed by Hoffmann et al.[26] and the NS DNA product was

individually cloned into pHW2000 plasmid vector. The NS recombinant plasmids together with the other seven recombinant plasmids carrying genomic segments of the PR8 virus in the TransIT®-LTI reagent (MirusBio, Madison, WI) were used to transfect the co-culture of MDCK and HEK-293 T cells. The inoculated culture was incubated at 37°C in a $CO_2$ incubator and daily observed for cytopathic effects. The NS reassortants were verified by sequencing, then propagated and titrated in MDCK cells by a plaque assay.

***Kinetics of virus replication in MDCK cells***

MDCK cell monolayers in 24 well tissue culture plates were infected with each reassortant virus at multiplicity of infection (m.o.i.) of 0.01. Virus adsorption was carried out for 2 hours at 37°C. Thereafter, the infected cell monolayers were washed twice with 1X EMEM and added with 1 ml of VGM per well and incubated at 37°C. The culture supernatants were harvested at day 1, 2, 3 and 5, and titrated for virus progenies by plaque assay in MDCK cell monolayers. In plaque assay, the culture supernatant was 10-fold serially diluted and inoculated onto confluent cell monolayers in 6-well tissue culture plates in triplicate. After viral adsorption for 90 minutes at 37°C, the infected cell monolayers were washed and maintained in semi-solid media containing 1.2% Avicel in EMEM and 2 µg/ml TPCK-trypsin. After incubation for 72 hours at 37°C, the cell monolayers were fixed with 10% formalin and stained with 1% crystal violet, then the number of plaques was counted.

***Virus infection in monocytic cell lines***

THP-1 and U937 cells were infected with each reassortant virus at the m.o.i. as indicated for each experiment. Virus adsorption was carried out for 2 hours at 37°C before washing to remove the virus inoculum. Thereafter, the infected cell cultures were resuspended in 1X RPMI supplemented with 2% FBS and 1 µg/ml TPCK-trypsin, followed by incubation at 37°C. At 24 hours post-infection, the cells were assessed for the expression of influenza NP and cytokines by flow cytometry.

***Flow cytometry***

Flow cytometry was used to assess the infected cells that expressed $NP^+$, $NP^+IFN\text{-}\alpha^+$, $NP^+IL\text{-}1\beta^+$ or $NP^+TNF\text{-}\alpha^+$. Briefly, $10^6$ monocytic cells were infected with rg-virus for 24 hours, and then fixed with 2% paraformaldehyde (PFA) for 10 minutes on ice, followed by cell permeabilization with 0.5% Tween-20 in phosphate buffered saline for 30 minutes at room temperature. The processed cells were stained with a mouse monoclonal antibody to influenza A NP (Millipore Corporation, Temecula, CA) for 1 hour on ice, washed with 1X PermWash (BD Biosciences) and then stained with fluorescein isothiocyanate (FITC) conjugated-goat anti-mouse Ig (BD Biosciences) in the dark for 1 hour on ice followed by washing with 1X PermWash. Thereafter, the cell pellets were suspended and added with antibody cocktail containing phycoerythrin (PE) conjugated- mouse anti-human IFN-α (MiltenyiBiotec, GmbH, Germany), peridinin chlorophyll protein/cyanin 5.5 complex (PerCP/Cy5.5) conjugated-mouse anti-human TNF-α (BioLegend Inc., San Diego, CA) and Alexafluor® 647 (AF647) conjugated-mouse anti-human IL-1β (BioLegend). The reaction tubes were kept on ice for an hour in the dark. The stained cells were washed twice, resuspended in 1% PFA and kept in the dark at 4°C until analyzed using a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed using FlowJo software (version 10.1) (Tree Star, Ashland, OR).

***Statistical analysis***

The numbers of plaques or numbers of stained cells were shown as mean ± standard deviations (SD). Data were plotted using GraphPad Prism version 5.0 (GraphPad software Inc., La Jolla, CA). Analysis of variance (ANOVA) was used to analyze the datasets. A $p$-value of $< 0.05$ was considered statistically significant.

## Results

***Phylogenetic analysis of NS genomic segments***

NS nucleotide sequences of the five study viruses were analyzed against the other 96 NS sequences belonging to 10 lineages (I-X) under alleles A and B[17] in order determine their genetic relationship. Phylogenetic trees were constructed by the neighbor-joining method using MEGA software version 5.2. The results demonstrate that the NS sequences of all study viruses belonged to allele A (**Figure 1A and B**). The NS sequences of PR8 (H1N1) and A/Thailand/Siriraj-02/2006 (H1N1) were clustered together in lineage I (Human H1N1 group), and the A/Prachuap Khiri Khan/137/2013 (H3N2) virus was also clustered in lineage I (human H3N2 group), showing a genetic relationship to the representative NS sequence of the A/Brevig Mission/1/18 (H1N1) virus. The NS sequence of the A/Thailand/104/2009 (H1N1) virus was clustered with the virus members in lineage II, showing a relationship with the representative NS sequence of A/Texas/05/2009 (H1N1) (**Figure 1A**). Based on the nucleotide sequences of the *HA* gene, H5N1 HPAI viruses were divided into 10 clades (clades 0-9). A detailed analysis demonstrated that the NS sequences of all 10 clades including the A/Thailand/1(KAN-1A)/2004 (H5N1) virus were clustered together in lineage VII (Eurasian /Oceanian) (**Figure 1B**). It is interesting that a clade 0 virus emerged in Hong Kong in 1997 was clustered in the same lineage as the clade 1-9 viruses that re-emerged after 2003, suggesting conservation of NS sequences. Nevertheless, their ancestor, A/goose/Guangdong/1/1996 (H5N1) belonged to lineage X of allele B. The results show that the NS nucleotide sequence of each study virus could be representative of its lineage/group.

***Alignment of NS1 and NS2 amino acid sequences***

The NS1 and NS2 amino acid sequences of the study viruses were aligned in order to determine the determinants of viral pathogenicity and virulence. NS1 protein is composed of approximately 207 to 237 amino acids, while NS2 protein is composed of about 121 amino acids.[13] Alignment of the NS1 and NS2 amino acid sequences of the study viruses were performed using BioEdit Sequence Alignment Editor version 7.2.5. Based on previous reports,[11,16,27-29] the key amino acid position(s) responsible for viral pathogenicity or sensitivity to IFN are highlighted (**Figure 2A**). The results show that these amino acid residues are located in NS1, but not in the NS2

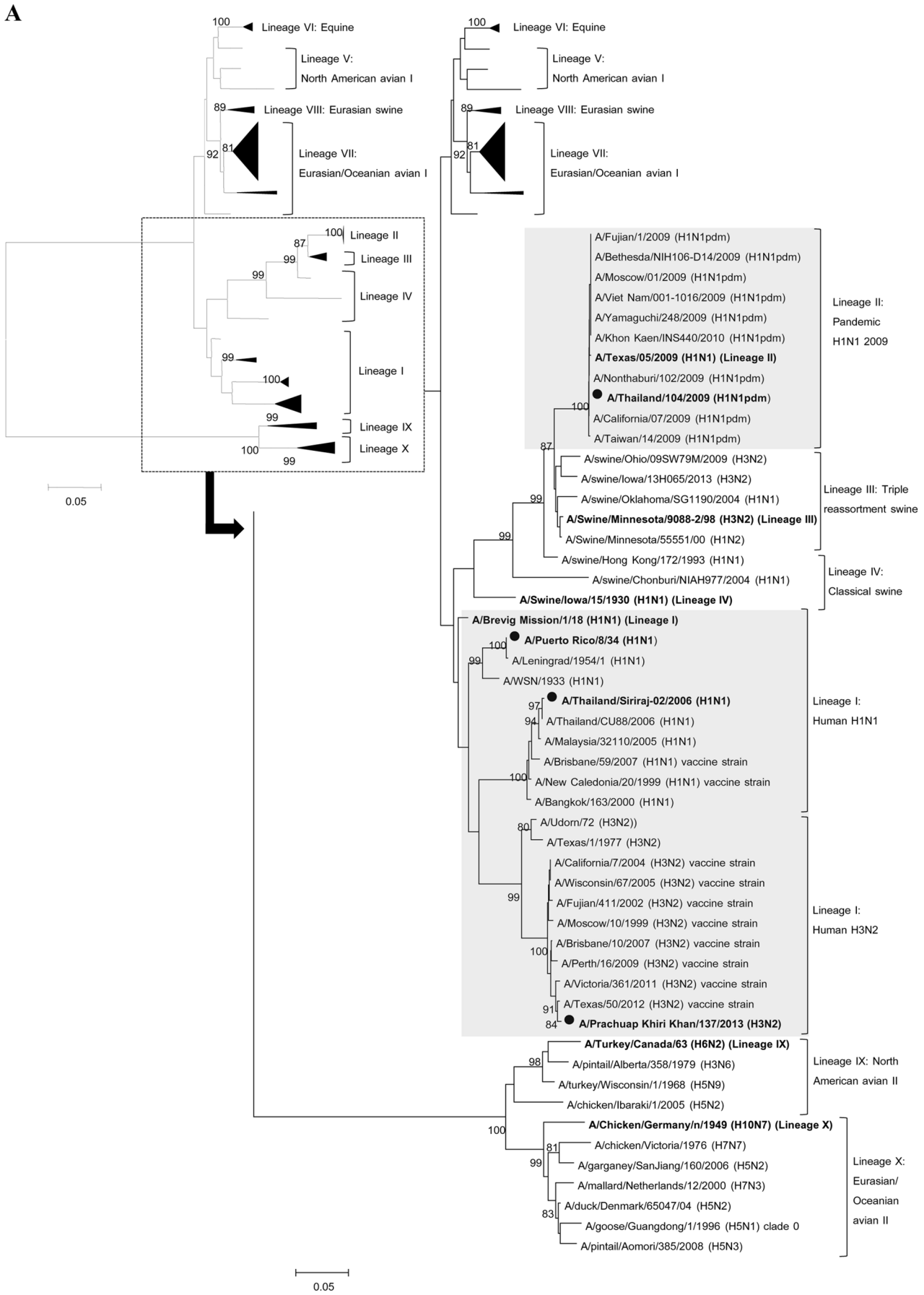


**Figure 1. Phylogenetic relationship of 101 influenza A NS nucleotide sequences comprising NS sequences from the viruses in this study and genetically distinct NS nucleotide pools from 10 lineages.** The nucleotide sequences of NS genomic segments were analyzed by phylogenetic tree using MEGA software version 5.2. The evolutionary distances are estimated by using neighbor-joining method and maximum composite likelihood algorithm. The reliability of the neighbor-joining tree is estimated by bootstrapping

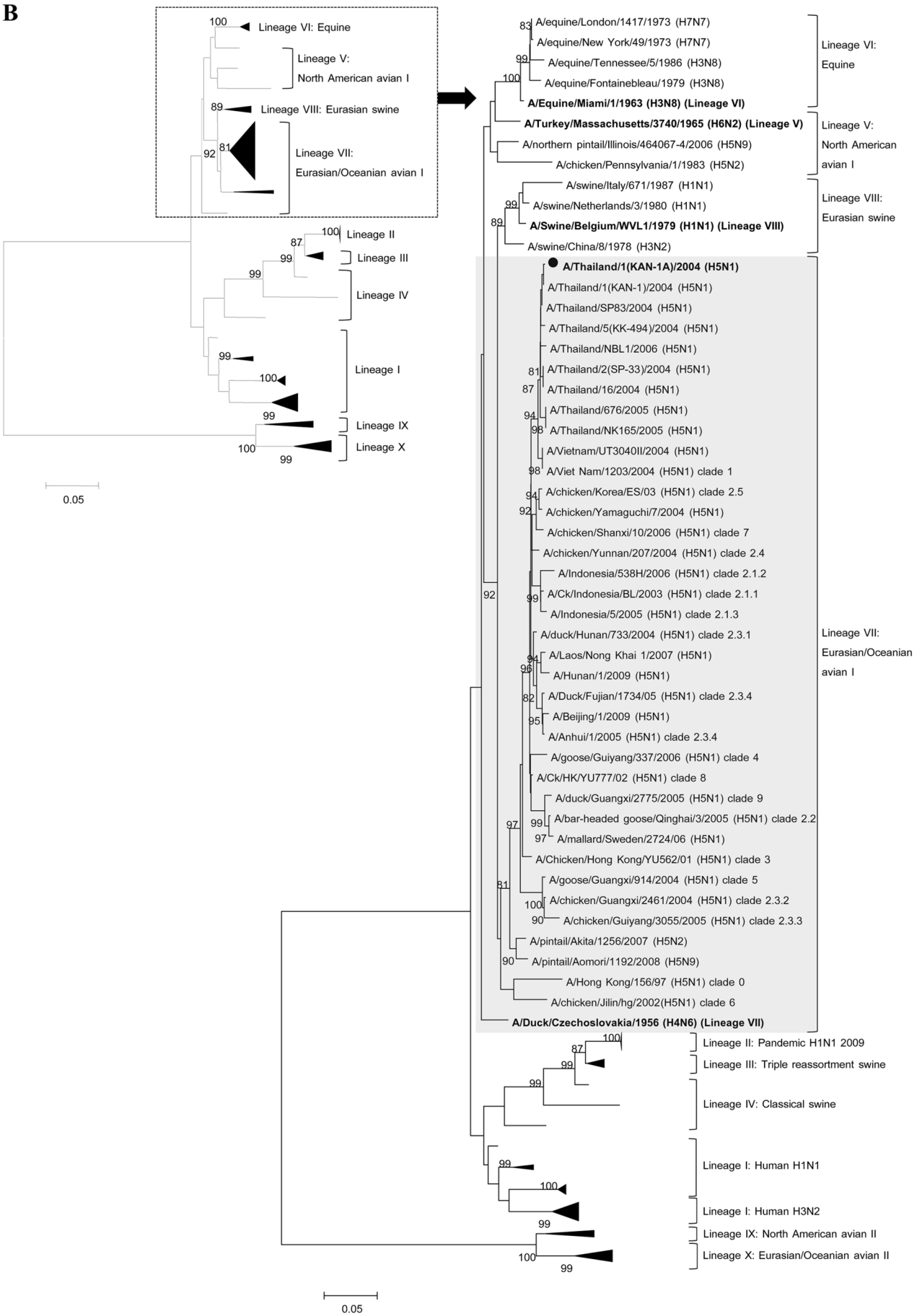


**Figure 1. (Continues)** analysis using 1,000 replicate datasets; and the percentages of bootstrap value of >80% are indicated at the nodes. The representative NS sequence of each lineage is bolded; while the virus names in this study are bolded with black circle ( ● ). Phylogenetic trees show detailed analysis of NS lineages I-IV, IX and X (A); and NS lineages V-VIII (B).

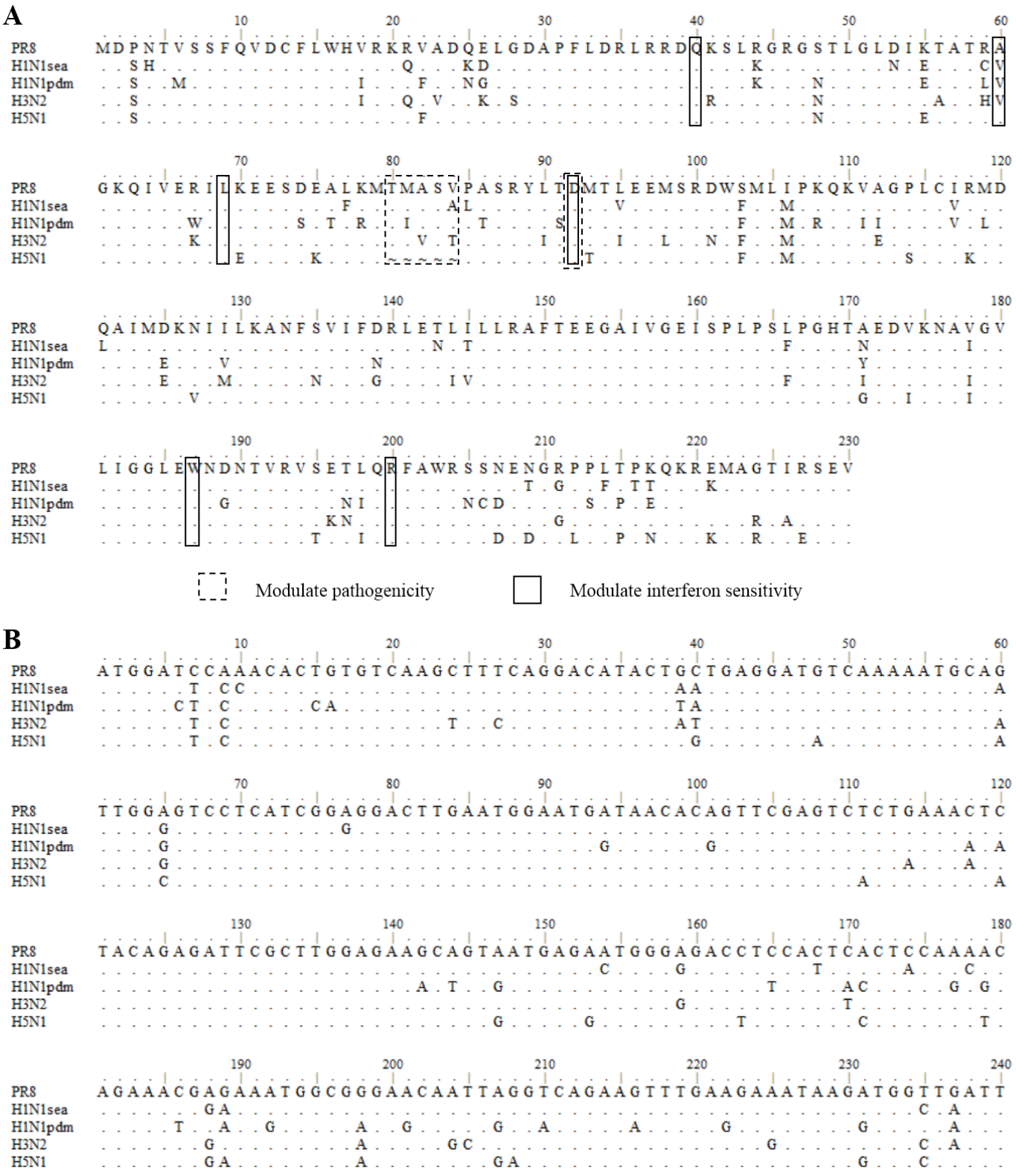


**Figure 2. Alignment of NS1 and NS2 amino acid sequences of the study viruses. A: NS1; B: NS2**

region (**Figure 2B**). Previous studies revealed that the D92E substitution[11] or five amino acid deletions at positions 80-84[16] in the NS1 protein could enhance the pathogenicity of H5N1 virus. However, only five amino acid deletions at position 80-84 were found in H5N1 NS in this study. None of the amino acid substitutions, i.e. Q40K, A60G, L69R, D92Y, W187C or R200I[27-29] known to modulate interferon sensitivity were present in the NS1 sequences of our viruses.

***Kinetics of replication of reassortant viruses in MDCK cells***

Two independent experiments on the kinetics of replication of the rg-viruses were performed in MDCK cells to demonstrate their efficiency of replication. The culture supernatants collected at days 1, 2, 3 and 5 post-infection (p.i.) were titrated by

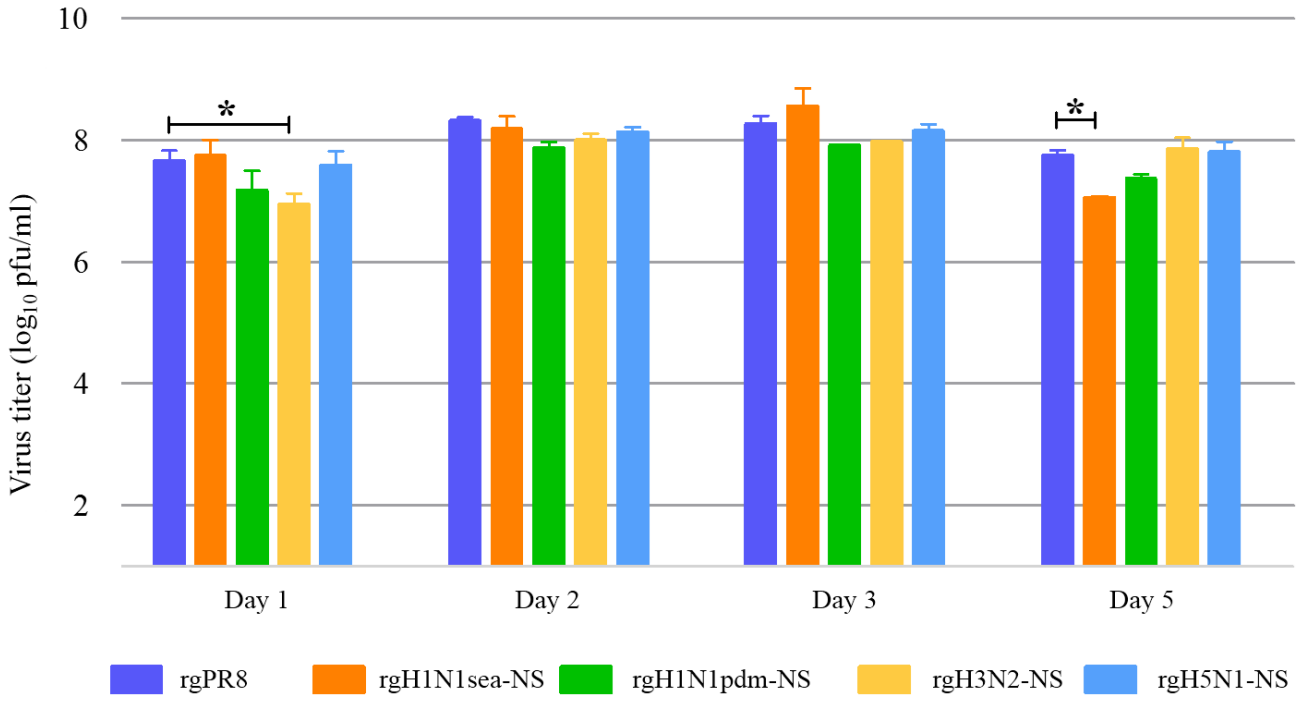


**Figure 3. Kinetics of replication of influenza reassortant viruses in MDCK cell monolayers at an m.o.i. of 0.01.** The culture supernatants are collected at indicated time points and titrated by plaque assay. Graphs show mean ± SD of duplicate experiments, and each experiment is performed in triplicate wells. Data is statistically analyzed by ANOVA (*, $P<0.05$).

the plaque assay in triplicate wells, and the average number of plaques ± SD for each dataset is shown in **Figure 3**. Even though these rg-viruses yielded different viral titers at days 1 and 5 p.i., all of them yielded comparable virus titers at their peaks of replication on days 2 or 3 p.i. (ANOVA; $P>0.05$), suggesting that these viruses replicated efficiently.

***Susceptibility of monocytic cells to reassorted PR8 virus***

Monocytes are known to be a good cell type to assess cytokine production in response to influenza virus infection. However, it has never been reported that U937 or THP-1 monocytic cells are more productive. In this study, the susceptibility of U937 and THP-1 monocytic cells to rgPR8 virus infection was explored using an m.o.i. of 1, 2 or 3 for 24 hours in three independent experiments. The success of viral infection was demonstrated by the presence of influenza viral NP intracellularly as analyzed by flow cytometry. The results show that both U937 and THP-1 cells were susceptible to rgPR8 virus, but the infection rate in U937 was significantly higher than that in THP-1 cells at all m.o.i. investigated (**Figure 4**). The percentages of infected U937 cells that expressed NP ($NP^+$ cells) were 6.8, 19.9 and 48.8% at an m.o.i. of 1, 2 and 3 (**Figure 4A**) compared to 0.06, 0.2 and 0.9% of infected THP-1 cells (**Figure 4B**), respectively. This difference was statistically significant (**Figure 4C**, ANOVA; $P<0.001$). Owing to the difference in susceptibility to viral infection, U937 cells were chosen for further study.

***Influence of distinct NS genomic segments on viral infectivity and cytokine induction***

The rg-viruses with distinct NS genomic segments were explored for their infectivity and cytokine induction in U937 monocytic cells using an m.o.i. of 3 in three independent experiments. At 24 hours p.i., the U937 cultures were analyzed regarding the number of infected cells ($NP^+$ cells) together with their expression of IFN-α ($NP^+IFN\text{-}\alpha^+$ cells), TNF-α ($NP^+TNF\text{-}\alpha^+$ cells) or IL-1β ($NP^+IL\text{-}1\beta^+$ cells) by flow cytometry. The results show that the average percentage of $NP^+$ cells induced by the rgPR8, rgH1N1sea-NS, rgH1N1pdm-NS, rgH3N2-NS or rgH5N1-NS viruses was 48.3, 46.8, 63.6, 46.7 or 76.1%, respectively. The number of $NP^+$ cells induced by the rgH5N1-NS virus was highest and significantly different from those produced by rgPR8, rgH1N1sea-NS and rgH3N2-NS (ANOVA; $P<0.05$), but not from rgH1N1pdm-NS virus (ANOVA; $P>0.05$) (**Figure 5A and B**). Nevertheless, the number of $NP^+$ cells induced by rgH1N1pdm-NS virus was not significantly higher than those induced by the rgPR8, rgH1N1sea-NS and rgH3N2-NS viruses.

It was found that most of the $NP^+$ cells produced IFN-α and far fewer produced IL-1β (**Figure 5A**). The rgH5N1-NS virus induced the highest numbers of $NP^+IFN\text{-}\alpha^+$ cells, followed by the rgH1N1pdm-NS virus, rgPR8, rgH1N1sea-NS and rgH3N2-NS viruses (**Figure 5C**). The number of $NP^+IFN\text{-}\alpha^+$ cells induced by the rgH5N1-NS was not statistically different from that induced by the rgH1N1pdm-NS virus (ANOVA; $P>0.05$), while it was significantly higher than those induced by the rgPR8, rgH1N1sea-NS and rgH3N2-NS viruses (ANOVA; $P<0.05$). Again, the number of $NP^+IFN\text{-}\alpha^+$ cells induced by rgH1N1pdm-NS virus was not significantly different from those induced by the rgPR8, rgH1N1sea-NS and rgH3N2-NS viruses. Furthermore, the numbers of $NP^+IL\text{-}1\beta^+$ cells produced by the five rg-viruses were not statistically different (ANOVA; $P>0.05$) (**Figure 5D**), and almost no $NP^+TNF\text{-}\alpha^+$ cells were produced by any of the rg-viruses investigated (**Figure 5A**).

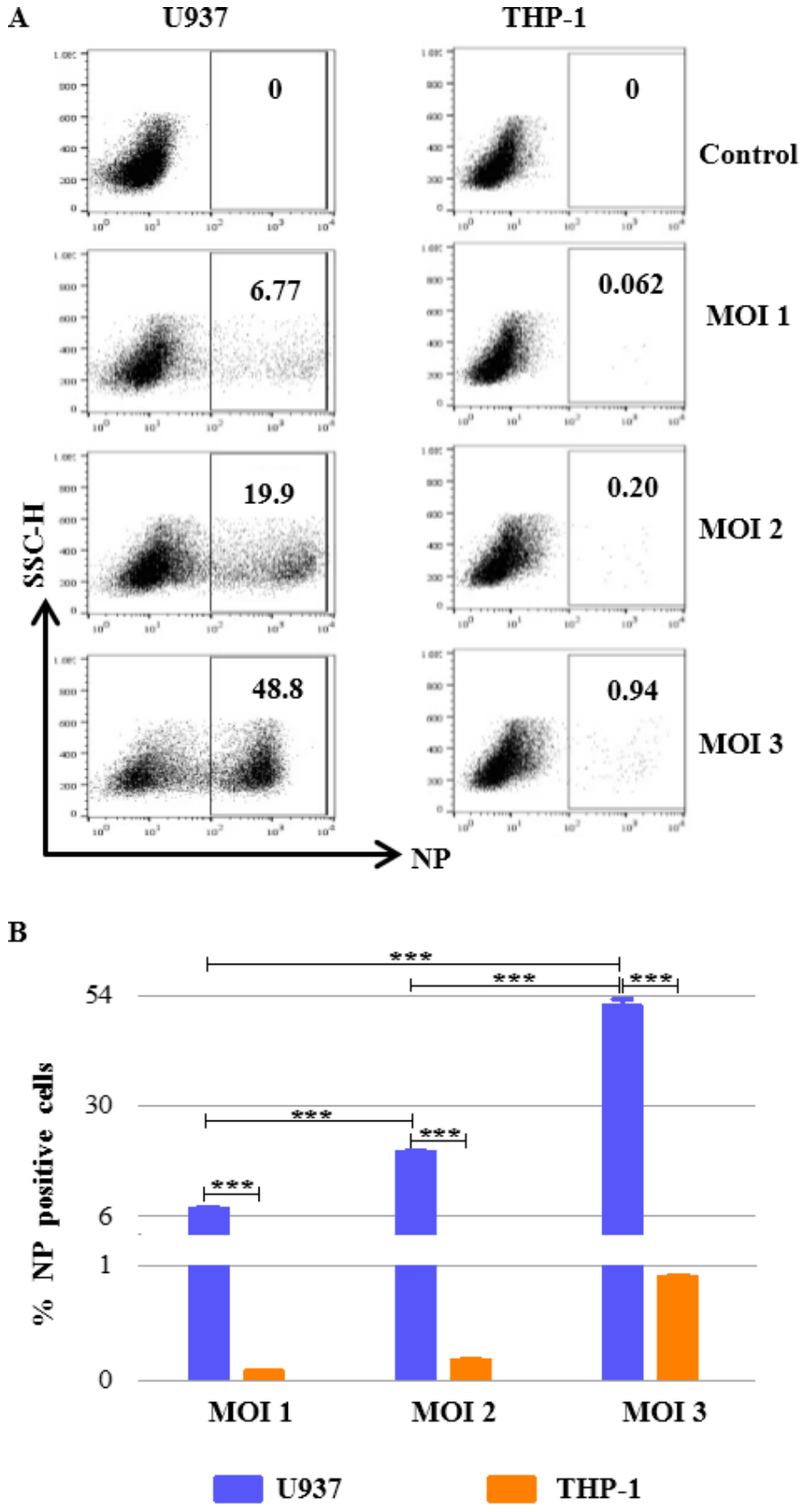


**Figure 4. Flow cytometry assay for determining the susceptibility of U937 or THP-1 monocytic cells to rgPR8 virus infection.** Three independent experiments are performed, and only one is shown as an example. Numbers indicate the percentages of cells in each gate. U937 monocytic cells (A); THP-1 monocytic cells (B). Average percentages (mean±SD) of $NP^+$ cells obtained from 3 independent experiments (C). U937 cells are significantly more susceptible to PR8 infection than THP-1 cells at all m.o.i. used (ANOVA: ***, $P<0.001$).

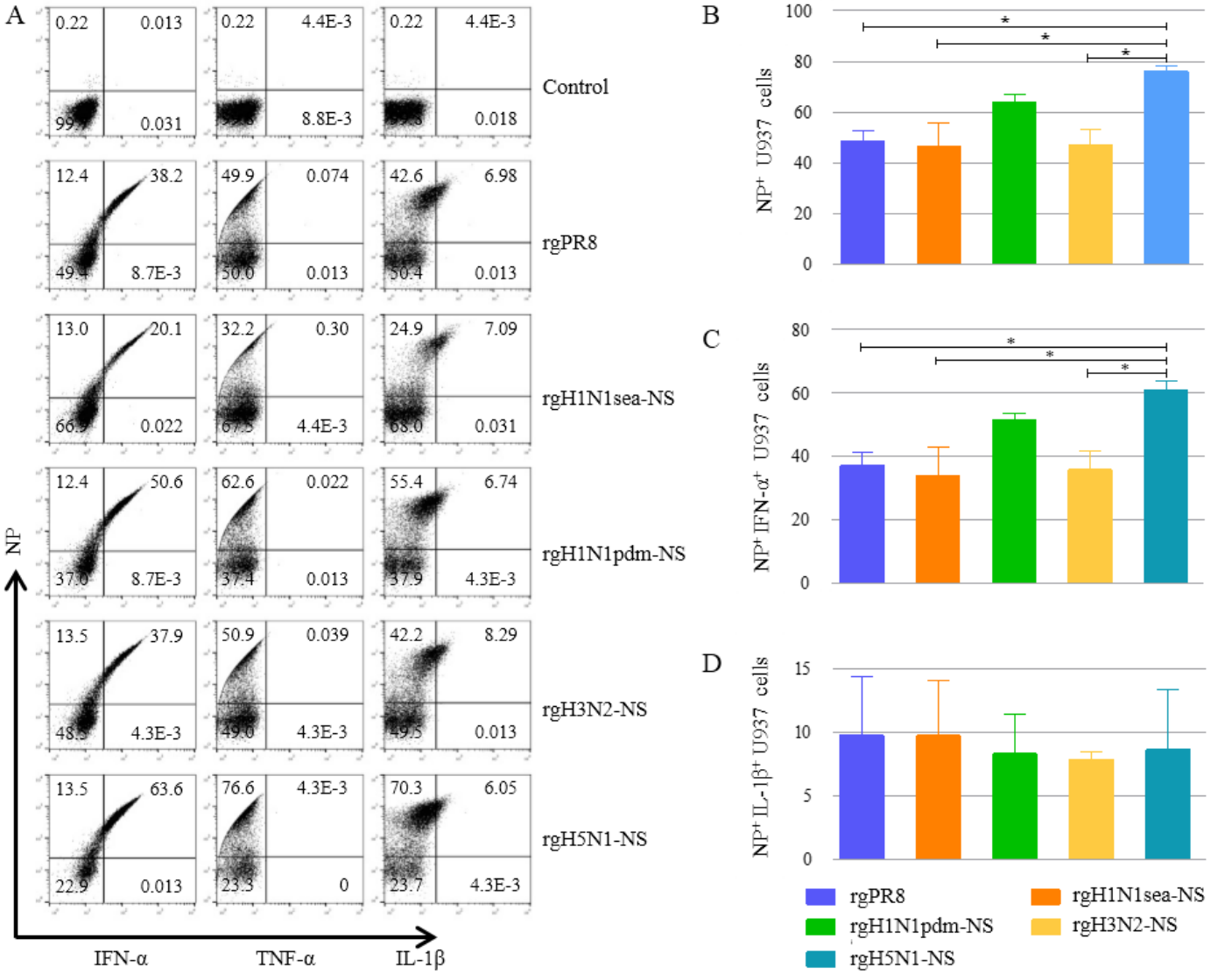


**Figure 5. Flow cytometry assay for determining viral infectivity and cytokine induction in U937 monocytic cells infected with NS reassortant viruses.** Three independent experiments are performed, and only one is shown as an example. X-axis shows numbers of cells positive for cytokines; and y-axis shows numbers of NP⁺ cells. Numbers indicate the percentages of cells in each gate. Uninfected cells are used as control staining (A); Histograms show the average percentages (mean ± SD) of stained cells from 3 independent experiments. NP⁺ cells (B); NP⁺IFN-α⁺ cells (C); NP⁺IL-1β⁺ cells (D). (ANOVA: *, $P<0.05$)

Collectively, these results demonstrate that the H5N1 NS genomic sequence significantly increased viral infectivity and cytokine induction compared to the other NS sequences.

## Discussion

This study constructed five rg-influenza viruses harboring distinct NS genomic segments derived from the H1N1sea, H1N1pdm, H3N2, H5N1 HPAI or PR8 virus to explore their influence on viral infectivity and the induction of cytokines in monocytic cells. The NS nucleotide sequences of these viruses belonged to distinct lineages and clustered together with members of the same subtype/group, i.e., the NS sequences of PR8 and H1N1sea belonged to lineage I (Human H1N1 group), H3N2 NS belonged to lineage I (Human H3N2 group), H1N1pdm NS belonged to lineages II and the NS sequences of all 10 clades of H5N1 viruses circulating after 1997 belonged to lineage VII, while that of A/goose/Guangdong/1/1996 (H5N1) belonged to lineage X. The phylogenetic analysis suggested that each virus in this study could be a representative of members of the same NS lineage.

It has frequently been found that the constructed reassortant viruses grow poorly and cannot be maintained in laboratories. However, the NS reassortants in this study were found to replicate efficiently and exhibited similar patterns of replication kinetics in MDCK cells. The NS1 and NS2 amino acid sequences of the study viruses were aligned and observed for the residues or regions previously known to influence viral pathogenicity and cytokine susceptibility (**Figure 2**). The results show that those determinants were located only in the NS1 sequence. The results suggest that the NS1 region plays a more important role in viral pathogenicity than the NS2 region. A deletion of five amino acids at positions 80-84 was found in H5N1 NS only. This deletion has been shown to enhance the virulence of the H5N1 HPAI virus in chickens.[16] In contrast, the D92E substitution

in the NS1 protein of the H5N1 HPAI virus, which had been shown to confer resistance to the antiviral action of IFN,[11] and the six amino acid substitution positions (Q40K, A60G, L69R, D92Y, W187C, R200I) which had been proposed to regulate interferon sensitivity[27-29] were not found in the study viruses.

It was found that U937 cells were much more susceptible than THP-1 cells against rgPR8 virus infection at all m.o.i. investigated, and the highest number of infected cells ($NP^+$ cells) was obtained at an m.o.i. of 3, as analyzed by flow cytometry. This difference might be due to the origin and maturation stage of both cell types. It was previously shown that human MDMs were more susceptible to influenza virus infection than blood monocytes.[30] On the other hand, THP-1 but not U937 cells constitutively express high amounts of interferon induced transmembrane protein 3 (IFITM3),[31] a molecule known to restrict influenza virus infection.[32] This result suggests that IFITM3 might be a cellular factor that determines susceptibility to influenza virus infection in this study.

The present study demonstrated that the rgH5N1-NS virus yielded the highest numbers of infected U937 cells compared to the other NS reassortant viruses. Most of the infected cells produced IFN-α and fewer cells produced IL-1β, while rare numbers produced TNF-α. Enhanced production of type I IFN had been suggested to be a pathogenic mechanism responsible for severe immunopathology in H5N1 HPAI.[33] However, results regarding the level of expression of TNF-α in H5N1 HPAI virus-infected cells are controversial among several groups of investigators. Similarly, our colleagues did not show overexpression of TNF-α in primary human macrophages infected with H5N1 HPAI isolates, compared to seasonal influenza H3N2 virus.[34] On the other hand, other studies have reported increased expression of TNF-α in human MDMs infected with rg-virus harboring the NS genomic segment of the H5N1 HPAI virus.[15,22] In patients infected with H5N1 HPAI virus, serum TNF-α concentrations have been found to be elevated,[7] or low,[6] or undetectable.[35]

The cytokine storm is known as a hallmark of patients infected with H5N1 HPAI virus compared to other influenza subtypes.[6,7] Using reverse genetics, the present study demonstrates that the H5N1 NS genomic segment plays a significant role in enhancing viral infectivity and IFN-α induction in monocytic cells. This finding is similar to results observed with wild type viruses in that the H5N1 HPAI virus induced higher levels of cytokines than H1N1pdm, sequentially followed by the H1N1sea and H3N2 viruses in MDMs.[20]

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## Competing interests

We declare no competing interests.

## Author Contributions

Conceived and designed the experiments: PPL, KB, PP; constructed the rg-viruses: PPL, DC; analyzed the NS sequences: PPL, DC, KS, PN; performed the experiments: PPL; analyzed the data: PPL, KB, PN, PP; contributed reagents/materials/analysis tools: KB, SP; prepared the manuscript: PPL, DC, PN, PP.

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