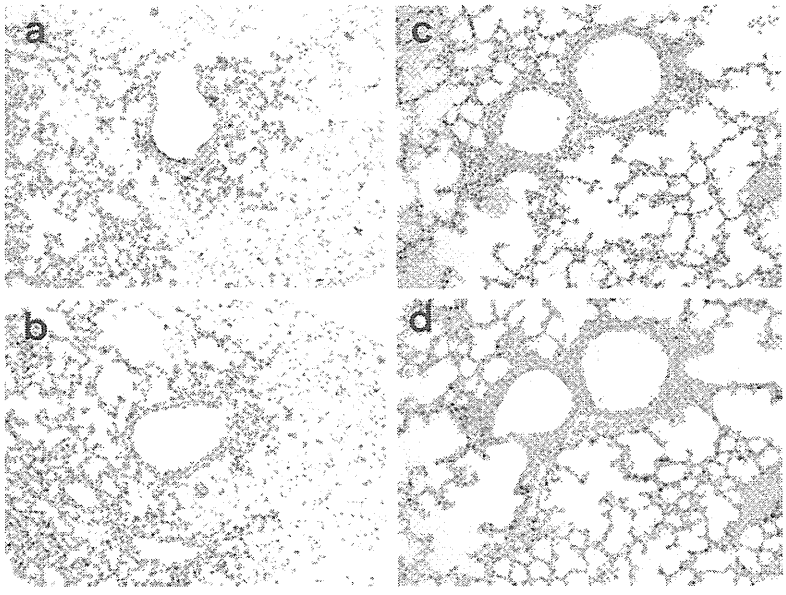


Fig. 3. Immunohistochemical analyses of lung sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483) virus. (a, b) Influenza virus antigens were detected in the epithelial cells of the bronchioles and alveoli of the mouse infected with A/Vietnam/1194/2004 (NIBRG-14) by the Niid_H5C clone (a) and polyclonal antibody against type A influenza nucleoprotein (b). (c, d) Virus antigens were not detected in the lung tissue section of the mouse infected with A/HongKong/483/97 (HK483) when Niid_H5C was used (c). However, virus antigens were detected in this section when a polyclonal antibody against type A influenza nucleoprotein was employed (d).

Table 2. Neutralizing ability of the eight mAbs generated in this study

| Clone | Neutralizing antibody titer (ng/mL) | | | |
|---|---|---|---|---|
| | NIBRG-14 (clade 1) | Indo-RG2 (clade 2.1) | NIBRG-23 (clade 2.2) | Anhui-RG5 (clade 2.3) |
| Niid_H5A | 78 | >10,000 | 625 | >10,000 |
| Niid_H5C | 625 | 625 | 313 | >10,000 |
| Niid_H5D | 625 | 625 | 313 | 5,000 |
| Niid_H5E | 625 | >10,000 | >10,000 | >10,000 |
| Niid_H5F | 313 | 313 | 156 | 2,500 |

| Test no. | Virus infection index ($Log_{10}$ $TCID_{50}$/0.05 mL) | | | |
|---|---|---|---|---|
| | NIBRG-14 | Indo-RG2 | NIBRG-23 | Anhui-RG5 |
| 1 | 2.5 | 3.1 | 2.4 | 2.1 |
| 2 | 2.0 | NT | 2.0 | 2.4 |

The in vitro neutralization assay examined the ability of the mAbs to neutralize H5N1 virus infection of cultured MDCK cells. Briefly, purified H5N1 virus was diluted to 2–3 × $10^2$ $TCID_{50}$/0.05 mL (the quantities are shown in the lower table) and incubated with serially-titrated purified mAbs for 1 h at 37°C. The samples were then placed into 96-well plates in which MDCK cells had been grown to 90% confluence. After 48 h, the cytotoxicity of the mAb-treated viruses was visualized by staining the cells with crystal violet.
NT, not tested.

dependency, suggesting that the epitopes of these mAbs differ. Interestingly, the mAbs were least able to neutralize Anhui-RG5. This may reflect the genetic distance between Anhui-RG5 (clade 2.3) and NIBRG-14 (clade 1).

**Antigen-capture ELISA:** To quantitatively detect H5N1 virus, we constructed a sandwich ELISA-based virus antigen-capture detection system. Preliminary experiments tested all combinations of two mAbs from the eight mAbs; Niid_H5F had the highest detection sensitivity for purified H5N1 virion and reacted broadly to the H5_HA of viruses belonging to clades 1, 2.1, 2.2, and 2.3. Therefore, Niid_H5F was selected as the antigen-capturing mAb. The antigen-capture ELISA was constructed by immobilizing Niid_H5F (and/or Niid_H5C) on the ELISA plate and using biotinylated Niid_H5D as the detection mAb, since this combination gave the best results (data not shown). Since the eight mAbs

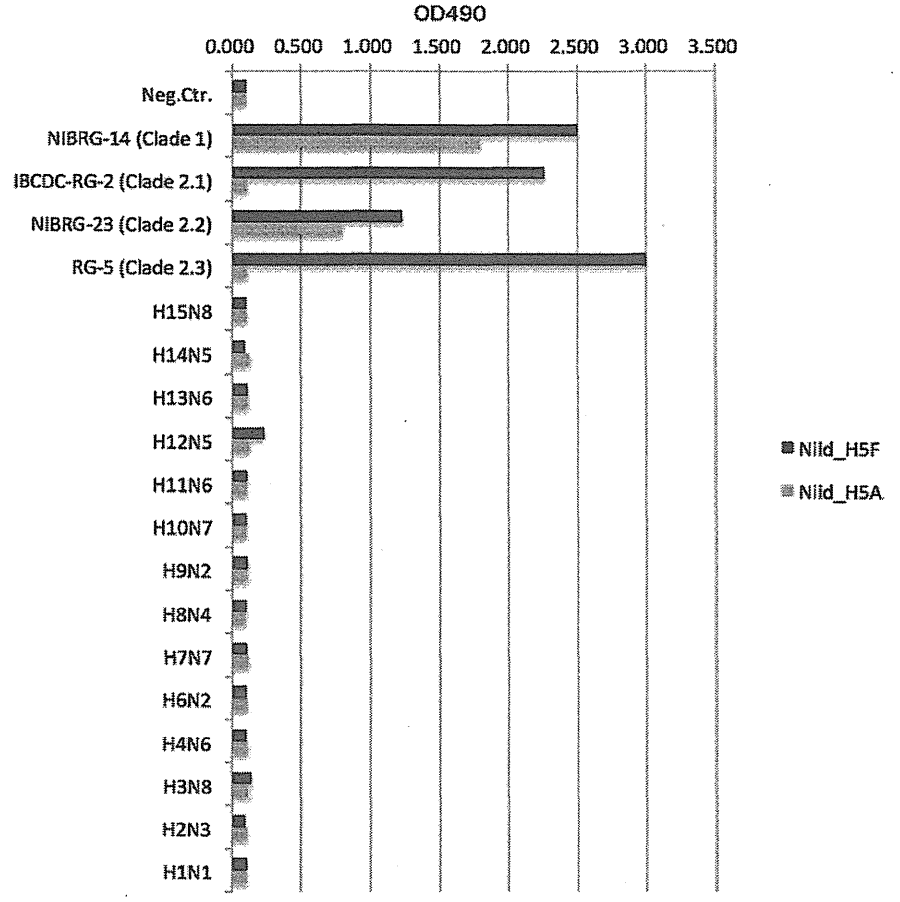


Fig. 4. ELISA reactivity of the Niid_H5A and Niid_H5F monoclonal antibodies (mAbs) to various influenza virus strains. Different influenza virus strains were immobilized on 96-well plates and incubated with biotinylated Niid_H5A or Niid_H5F mAbs followed by peroxidase-labeled streptavidin. The binding of the mAbs was then quantitated by a colorimetric assay using TMB as a substrate.

were originally raised against the H5N1 virus strain A/Vietnam/1194/2004 (NIBRG-14), the validity of this system with other strains of H5N1 virus was also examined. As shown in Fig. 4, this system could detect the A/Indonesia/05/2005 (Indo5/PR-8-RG2), A/Turkey/1/2005 (NIBRG-23), and A/Anhui/01/2005 (Anhui01/PR8-RG5) strains but none of the non-H5N1 strains. The sandwich ELISA could detect H5N1 virus protein at concentrations as low as 50 ng/mL HA, namely, $>3$ SD of negative samples (Fig. 5).

## DISCUSSION

In the present study, mAbs against H5N1 influenza virus were established. These mAbs could detect the virus when used in Western blot analyses, IFA, immunohistochemical analyses, neutralization assays, and antigen-capture ELISA. The characteristics of the mAbs are summarized in Table 1.

Of the eight mAb clones that reacted to H5N1 virus in ELISAs, six reacted to rHA. Only one clone reacted to NA protein. Another clone detected an unknown 150-kDa molecule upon Western blot analysis. A hybridoma that secreted a mAb that could detecte the nuclear protein or other protein components of H5N1 virus was not detected, presumably because the first screening step identified H5 specificity. These results indicate that the HA protein is a dominant target in the antibody response of HA-subtype specificity, as suggested by other studies (17,18). There is accumulating evidence that the influenza strain-specific epitopes are often localized on the HA1 region, whereas the epitopes that are conserved among various strains are localized on the HA2 region (19–22). It has been reported that the immune response elicited by H1N1pdm yields a high frequency of HA2-specific mAbs (23,24). In the present study, none of the established clones detected the HA2 fragment of H5HA, presumably because this study focused on H5-specific clones.

The mAbs isolated in the present study were assessed for their ability to detect H5N1 virus-infected MDCK cells in IFA. Indeed, the anti-HA and anti-NA mAbs detected the cytoplasmic Golgi-rich region and the cell surface membrane. This reflects the common assembly process of influenza virus (25).

In general, a single diagnostic test is not reliable because of the potential for false positives and negatives. Considering the restricted availability of RNA detection systems (26,27), serological screening systems other than those that detect antibodies are currently being ex-

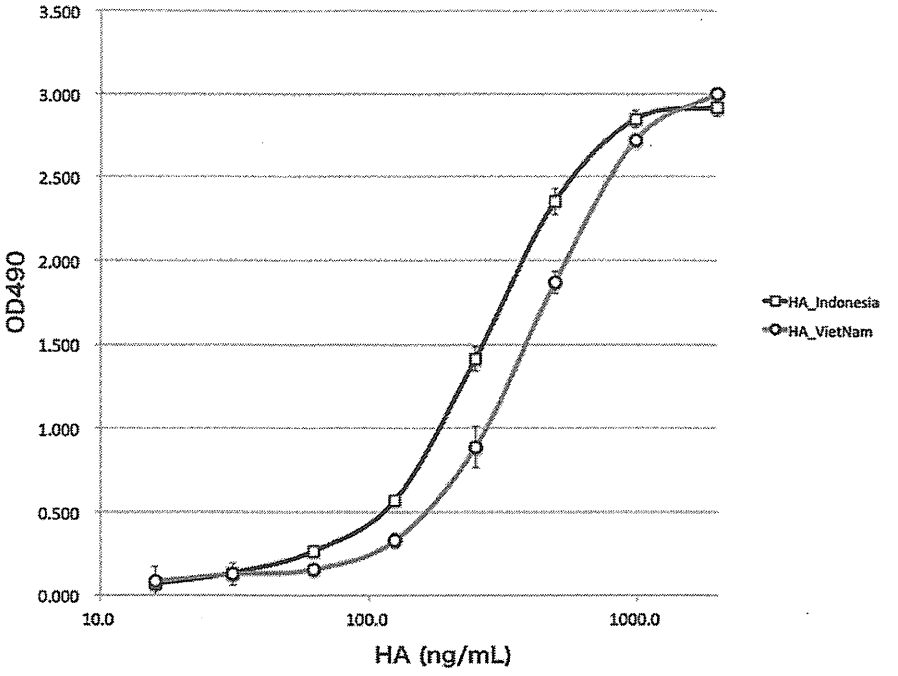


Fig. 5. Antigen-capture ELISA reactivity of monoclonal antibodies (mAbs) to H5N1 and H1N1 virus strains. The anti-H5 mAb Niid_H5F was immobilized on 96-well plates and reacted with serially-titrated purified H5N1 virus fractions for 1 h at room temperature. The bound virus proteins were detected by incubation with biotinylated Niid_H5D (anti-H5) antibody followed by peroxidase-labeled streptavidin. The binding was quantitated by a colorimetric assay that used TMB as a substrate. Abscissa, concentration of purified H5N1 virus proteins. Ordinate, absorbance unit (OD490).

amined. ELISA-based antigen-capture assays offer high specificity and reproducibility and have been used to diagnose and monitor many diseases. The present study describes the development of an antigen-capture ELISA system that detects purified H5N1 virus virion at levels as low as 50 ng/mL. The sensitivity of this system, which comprises three anti-HA mAbs, appears sufficiently high to detect virus protein in patient sera, particularly since a recently reported antigen-capture ELISA system detects 50 ng/mL of purified recombinant HA1 protein (28). At present, the sensitivity of the system is being improved, and its usefulness in diagnosing and monitoring H5N1 virus infections is being validated.

The five selected anti-HA mAbs exhibited significant neutralization activity against several viral strains in a clade-dependent manner (Table 2). Of these, Niid_H5F showed the broadest spectrum of neutralization activity, but it neutralized NIBRG-23 (clade 2.2) more efficiently than the original immunogen NIBRG-14 (clade 1). It would be of interest to determine the features that determine this clade-dependency of mAb recognition. It is also possible that these mAbs have therapeutic potential, if humanized by means of complementarity determining region grafting or mouse-human chimerism.

In conclusion, eight new H5N1-specific mAbs were generated from A/Vietnam/1194/2004 (NIBRG-14)-hyperimmunized mice, six of which were HA-specific. These mAbs were useful in Western blot analyses, IFA, and immunohistology and had in vitro neutralization activity against H5N1 viruses. These mAbs also perform well in a highly sensitive antigen-capture sandwich ELISA system. As such, these mAbs may be useful for the rapid and specific diagnosis of H5N1 subtype influenza virus and may have therapeutic potential.

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**Conflict of interest** None to declare.

## REFERENCES

1. Chan, M.C., Cheung, C.Y., Chui, W.H., et al. (2005): Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir. Res., 6, 135.
2. World Health Organization Global Influenza Program Surveillance Network (2005): Evolution of H5N1 avian influenza viruses in Asia. Emerg. Infect. Dis., 11, 1515–1521.
3. Webster, R.G. and Govorkova, E.A. (2006): H5N1 influenza—continuing evolution and spread. N. Engl. J. Med., 355, 2174–2177.
4. Webster, R.G., Guan, Y., Peiris, M., et al. (2002): Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern china. J. Virol., 76, 118–126.
5. World Health Organization: Online at 〈http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_2008_2019/en/index.html〉.
6. Uyeki, T.M. (2009): Human infection with highly pathogenic avian influenza A (H5N1) virus: review of clinical issues. Clin. Infect. Dis., 49, 279–290.

7. Gambotto, A., Barratt-Boyes, S.M., de Jong, M.D., et al. (2008): Human infection with highly pathogenic H5N1 influenza virus. Lancet, 371, 1464–1475.
8. Abdel-Ghafar, A.N., Chotpitayasunondh, T., Gao, Z., et al. (2008): Update on avian influenza A (H5N1) virus infection in humans. N. Engl. J. Med., 358, 261–273.
9 de Jong, M.D., Simmons, C.P., Thanh, T.T., et al. (2006): Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat. Med., 12, 1203–1207.
10. Centers for Disease Control and Prevention: Seasonal Influenza (Flu). Influenza Diagnostic Testing Algorithm. Online at ⟨http: // www.cdc.gov / flu / professionals / diagnosis / testing_ algorithm.htm⟩.
11. Wright, P.F., Neumann, G. and Kawaoka, Y. (2007): Orthomyxoviridae. Lippincott Williams & Wilkins, Philadelphia.
12. World Health Organization: Influenza Continuing Progress towards a Unified Nomenclature System for the Highly Pathogenic H5N1 Avian Influenza Viruses. Online at ⟨http://www.who.int/influenza/resources/documents/h5n1_nomenclature/en/⟩.
13. Takahashi, Y., Hasegawa, H., Hara, Y., et al. (2009): Protective immunity afforded by inactivated H5N1 (nibrg-14) vaccine requires antibodies against both hemagglutinin and neuraminidase in mice. J. Infect. Dis., 199, 1629–1637.
14. Kozbor, D. and Roder, J.C. (1984): In vitro stimulated lymphocytes as a source of human hybridomas. Eur. J. Immunol., 14, 23–27.
15. Storch, G.A. (2001): Diagnostic virology. p. 493–531. Lippincott Williams & Wilkins, Philadelphia.
16. Ohnishi, K., Sakaguchi, M., Kaji, T., et al. (2005): Immunological detection of severe acute respiratory syndrome coronavirus by monoclonal antibodies. Jpn. J. Infect. Dis., 58, 88–94.
17. Graves, P.N., Schulman, J.L., Young, J.F., et al. (1983): Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. Virology, 126, 106–116.
18. Russ, G., Polakova, K., Kostolansky, F., et al. (1987): Monoclonal antibodies to glycopolypeptides HA1 and HA2 of influenza virus haemagglutinin. Acta Virol., 31, 374–386.
19. Kashyap, A.K., Steel, J., Oner, A.F., et al. (2008): Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc. Natl. Acad. Sci. USA, 105, 5986–5991.
20. Throsby, M., van den Brink, E., Jongeneelen, M., et al. (2008): Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IGM$^+$ memory B cells. PLoS One, 3, e3942.
21. Sui, J., Hwang, W.C., Perez, S., et al. (2009): Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol., 16, 265–273.
22. Ekiert, D.C., Bhabha, G., Elsliger, M.A., et al. (2009): Antibody recognition of a highly conserved influenza virus epitope. Science, 324, 246–251.
23. Corti, D., Suguitan, A.L., Jr., Pinna, D., et al. (2010): Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest., 120, 1663–1673.
24. Wrammert, J., Koutsonanos, D., Li, G.M., et al. (2011): Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J. Exp. Med., 208, 181–193.
25. Palese, P. and Shaw, M.L. (2007): Orthomyxoviridae: the viruses and their replication. p. 1647. Lippincot Williams & Wilkins, Philadelphia.
26. Imai, M., Ninomiya, A., Minekawa, H., et al. (2007): Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method. J. Virol. Methods, 141, 173–180.
27. Wei, H.L., Bai, G.R., Mweene, A.S., et al. (2006): Rapid detection of avian influenza virus a and subtype H5N1 by single step multiplex reverse transcription-polymerase chain reaction. Virus Genes, 32, 261–267.
28. He, Q., Velumani, S., Du, Q., et al. (2007): Detection of H5 avian influenza viruses by antigen-capture enzyme-linked immunosorbent assay using H5-specific monoclonal antibody. Clin. Vaccine Immunol., 14, 617–623.

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ORIGINAL ARTICLE

# Key role of regulated upon activation normal T-cell expressed and secreted, nonstructural protein1 and myeloperoxidase in cytokine storm induced by influenza virus PR-8 (A/H1N1) infection in A549 bronchial epithelial cells

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## ABSTRACT

**Influenza virus infection causes severe respiratory disease such as that due to avian influenza (H5N1). Influenza A viruses proliferate in human epithelial cells, which produce inflammatory cytokines/chemokines as a "cytokine storm" attenuated with the viral nonstructural protein 1 (NS1). Cytokine/chemokine production in A549 epithelial cells infected with influenza A/H1N1 virus (PR-8) or nonstructural protein 1 (NS1) plasmid was examined *in vitro*. Because tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and regulated upon activation normal T-cell expressed and secreted (RANTES) are predominantly produced from cells infected with PR-8 virus, the effects of mRNA knockdown of these cytokines were investigated. Small interfering (si)TNF-$\alpha$ down-regulated RANTES expression and secretion of RANTES, interleukin (IL)-8, and monocyte chemotactic protein-1 (MCP-1). In addition, siRANTES suppressed interferon (IFN)-$\gamma$ expression and secretion of RANTES, IL-8, and MCP-1, suggesting that TNF-$\alpha$ stimulates production of RANTES, IL-8, MCP-1, and IFN-$\gamma$, and RANTES also increased IL-8, MCP-1, and IFN-$\gamma$. Furthermore, administration of TNF-$\alpha$ promoted increased secretion of RANTES, IL-8, and MCP-1. Administration of RANTES enhanced IL-6, IL-8, and MCP-1 production without PR-8 infection. These results strongly suggest that, as an initial step, TNF-$\alpha$ regulates RANTES production, followed by increase of IL-6, IL-8, and MCP-1 and IFNs concentrations. At a later stage, cells transfected with viral NS1 plasmid showed production of a large amount of IL-8 and MCP-1 in the**

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**List of Abbreviations:** ARDS, acute respiratory distress syndrome; DMEM, Dulbecco's modified Eagle's minimal essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL-6R, IL-6-recepter; IP-10, interferon-$\gamma$-induced protein 10; MCP-1, monocyte chemotactic protein 1; M-CSF, macrophage colony-stimulating factor; MIG, monokine induced by interferon $\gamma$; MPO, myeloperoxidase; MTT, thiazolyl blue tetrazolium bromide; NHP, National Hospital of Pediatrics; NPA, nasopharyngeal aspirate; NS1, nonstructural protein 1; pfu, plaque-forming units; qPCR, quantitative polymerase chain reaction; RANTES, regulated upon activation normal T-cell expressed and secreted; rRANTES, recombinant RANTES; rTNF-$\alpha$, recombinant TNF-$\alpha$; sIL-6R, soluble interleukin 6 receptor; siRNA, small interfering RNA; TNF-$\alpha$: tumor necrosis factor $\alpha$; TNFR2, tumor necrosis factor 2 receptor.

**presence of the $H_2O_2$-myeloperoxidse (MPO) system, suggesting that NS1 of PR-8 may induce a "cytokine storm" from epithelial cells in the presence of an $H_2O_2$-MPO system.**

**Key words** bronchial epithelial cells, influenza viral NS1, myloperoxidase, regulated upon activation normal T-cell expressed and secreted.

Influenza virus infection causes severe respiratory disease such as that caused by avian influenza virus A (H5N1), which produces a wide range of clinical manifestations, including asymptomatic infections, mild to severe respiratory disease, diarrhea, vomiting, abdominal and pleuritic pain in the early stages and severe disease with high morbidity and mortality (1). Since 2004, 10 young patients have died of A/H5N1 infection in Vietnam. Commonly occurring prognostic indicators for ARDS and death are leukocytopenia and thrombocytopenia (2, 3). Most patients with influenza virus infection of the H5N1 subtype manifest ARDS during their clinical course, often followed by deterioration and death from respiratory failure. The histopathology of these cases confirms that ARDS is a disorder of the respiratory system (4). Indeed, the serious prognosis of these patients is due to the respiratory manifestations, because influenza viruses replicate in the epithelial cells of the respiratory tract (5). On the other hand, H5N1 influenza infection also results in systemic tissue damage mediated by immunological responses, inflammatory cytokine levels in the blood increasing dramatically during severe infections (6–8). In addition, other types of influenza virus infection cause reduced production of MCP-1, RANTES, IL-8, and IP-10 in the late stages of infection. Moreover, pretreatment with TNF-$\alpha$ or IFN-$\alpha$ greatly enhances influenza-A-virus-induced chemokine production (9). Thus, TNF-$\alpha$, IFN-$\alpha$, MCP-1, RANTES, and IL-8 play key roles in the early stages of influenza virus infection of epithelial cells. Thus, innate immunology responses occur when influenza viruses infect epithelial cells. Many studies using PR-8 have shown that cytokines are produced from human lung tissue models in response to influenza virus PR8 (H1N1) infection. In addition, influenza A/PR/8/34 virus infection results in significant induction of genes involved in the IFN pathway (10). Moreover, PR-8 influenza virus infection in cells rapidly activates mitogen activated protein kinase signaling *in vitro*. When influenza A virus replicates in epithelial cells, macrophages and leukocytes respond by producing chemokines and proinflammatory cytokines. Uncontrolled viral replication and the associated "cytokine storm" of IL-6, IL-8, IP-10, MIG, and MCP-1 is responsible for this infection's serious clinical manifestations and poor outcomes (6, 11, 12). In fact, our previous study showed that IL-12p40 and TNF-R2 in plasma and IL-6R in NPA increase in ARDS patients infected with influenza virus (13). In addition, H1N1 (PR-8)-virus-infected mice show severe ARDS in pulmonary epithelial cells.

Thus, virus-infected macrophages and dendritic cells can produce significant amounts of TNF-$\alpha$ and type I IFNs in response to influenza A infection. These cytokines may act locally in virus-infected tissues to enhance the expression of proteins involved in virus recognition and signal transduction. The cytokine priming leads to strong virus-induced activation of transcription factors and enhanced secondary cytokine and chemokine responses in later phases of influenza A virus infection (9). Type I IFNs and inflammatory cytokine expression are attenuated with viral NS1, which is a potent virulence factor for influenza A virus (14). The NS1 protein of influenza A virus is a multifunctional protein that contributes significantly to disease pathogenesis by modulating many virus and host-cell processes (15, 16). In addition, NS1 has the ability to limit IFN-$\beta$ induction by both pre-transcriptional and post-transcriptional nuclear processes (17). Recently, NS1 has been demonstrated to induce apoptosis of epithelial cells (18). Furthermore, MPO activity increases in the plasma of patients with influenza virus infection (13). Neutrophil-derived MPO in the inflammation of lung infected with influenza virus causes pulmonary pathology, in which recruitment and activation of neutrophils are associated with oxidative tissue damage (19).

In the present study, we examined the sequential order of the stream of cytokines and chemokines produced in A549 epithelial cells infected with PR-8 *in vitro*. In addition, we analyzed the role of neutrophil-derived MPO and NS1 of PR-8 in "cytokine storms" associated with influenza infection.

## MATERIALS AND METHODS

### Viruses

Human influenza A/(H1N1)PR-8 virus was originally obtained from the strain collection of the National Institute of Infectious Disease (Tokyo, Japan).

### Preparation of nonstructural protein 1 plasmid

The full length of cDNA encoding NS1 of PR-8 was amplified by primers combined with EcoRI and XhoI sites. The cDNA fragment was ligated into the pCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA), and DH5$\alpha$-T1$^R$ *Escherichia coli* (Invitrogen) was transformed with the vector for subcloning. The purified plasmid was treated with EcoRI and XhoI enzymes, and ligated with pCMV-myc vector (Clontech, Palo Alto, CA, USA) treated with same enzyme pair to create the pCMV-myc-NS1 construct. The construct was amplified with DH5$\alpha$-T1$^R$ *E. coli*, and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) after electrophoresis.

### Culture of human alveolar epithelial cell line A549

Human alveolar epithelial cell line (A549) cells were maintained in DMEM (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS (Gibco, Gaithersburg, MD, USA), 100 units/mL penicillin, and 100 units/mL streptomycin in tissue culture flasks (Corning, Cambridge, MA, USA) at 37°C in a 5% $CO_2$ incubator.

### Virus infection to A549 cells

Monolayers of A549 cells at a concentration of $1 \times 10^5$ cells/ml in 6-well plates incubated for 24 hr ($1 \times 10^6$) were infected with the viruses at 1000 pfu diluted in Opti-MEM (Invitrogen). This titer as pfu of the virus was employed for optimal minimization of the concentration into the A549 cells. After 1 hr the infective solution was removed; the cells washed twice with DMEM and supplemented with a growth medium of DMEM, 5% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin and incubated at 37°C in a 5% $CO_2$ incubator.

### Transfection of nonstructural protein 1 to A549 cells

A549 cells were transfected with pCMV-myc ligated with NS1 (pCMV-myc-NS1) at a concentration of 0.008 $\mu$g/mL in 3 mL of DMEM culture medium containing 1% of 100 units/mL penicillin and 100 units/mL streptomycin, and 5% FBS using lipofectamine 2000 reagent (Invitrogen). Transfection followed the procedure provided by the manufacturers: the cells were incubated for 6 hr after being washed twice with DMEM and replacement of the growth medium with DMEM, 5% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in a 5% $CO_2$ incubator. Production of NS1 in the cells transfected with NS1 plasmid was determined by mRNA expression.

### Transfection of small interfering RNA to A549 infected with PR-8

siRNA of the following eight cytokines: siIFN-$\alpha$, siIFN-$\beta$, siTNF-$\alpha$, siIL-1$\beta$, siIL-6, siIL-12p40, siRANTES, and siIL-5 (Sigma Genosys siRNA Service, Sapporo, Japan) were transfected into A549 cells using lipofectamine RNAiMAX (Invitrogen). After transfection of siRNA into A549 cells ($1 \times 10^6$ cells/mL) according to the standard procedure supplied by the manufacturers, they were incubated for 6 hr. The cells were then washed twice with DMEM and infected with PR-8 for 1 hr. Then the cells were washed twice with DMEM, placed in a growth medium of DMEM, 5% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin, and incubated at 37°C in a 5% $CO_2$ incubator. Cells and culture fluid were harvested at 2 and 4 days after infection.

### Administration of recombinant tumor necrosis factor-$\alpha$ and recombinant regulated upon activation normal T-cell expressed and secreted to uninfected-A549 cells

R tumor necrosis factor-$\alpha$ or rRANTES at a concentration of 10 ng/mL in Opti-MEM (Invitrogen) was added to the uninfected-A549 cells at a concentration of $1 \times 10^6$ cells/mL in 6-well plates. The cells were incubated for 1 hr and washed with DMEM. After further incubation in DMEM containing 5% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin for 2 days at 37°C in a 5% $CO_2$ incubator, the culture fluid was obtained from the wells.

### Administration of human myeloperoxidase to A549 cell culture infected with PR-8 or nonstructural protein 1

Human myeloperoxidase was isolated from neutrophils of volunteers as has been described elsewhere (20). After infection with PR-8 or transfection with NS1 plasmid, the A549 cells were cultured for 2 hr at 37°C in a 5% $CO_2$ incubator, then hMPO (1 and 3 units/mL) in PBS containing 0.001% BSA (ICN Biomedicals 81-028, Aurora, OH, USA) was added to the cells with $H_2O_2$ (0.01 mM in PBS). The cells and culture fluid were harvested at 2 and 4 days after infection.

### Polymerase chain reaction

Total RNA was extracted from the cells with Isogen (Nippon Gene, Toyama, Japan) and 1.0 $\mu$g of the total RNA was transcribed to first strand of cDNA using a Rever Tra Ace $\alpha$-First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan) in a total of 20 $\mu$L. Using a primer set for internal and target genes, the PCR was performed in 1.5 mM $MgCl_2$ and 0.2 pmol of primers and *Taq* DNA

Polymerase Hot Start (Takara, Kyoto, Japan) in a total reaction volume of 20 $\mu$L. The PCR used the following protocol: denaturation at 95°C for 3 min, 95°C for 30 sec, 60°C for 1 min, and 72°C for 5 min for 25–35 cycles, and then 4°C.

### Quantitative polymerase chain reaction analysis of gene expression by using SYBR Green

The differential expression data were validated by qPCR. One hundred nanograms of total RNA from control and infected A549 cells was used for qPCR analysis. All qPCR assays were performed in the same format and run on the StepOne Real-Time PCR System in a 48-well plate (ABI Life Technologies, Carlsbad, CA, USA). The reaction was performed from cDNA using the SYBR Green PCR Master Mix QuantiTect (ABI, Life Technologies) according to the manufacturer's instructions using primers (Supplemental Table 1). Reaction efficiency was calculated by using serial 10-fold dilutions of the housekeeping gene encoding GAPDH and sampled with identical cycle conditions: 95°C for 5 min, and 45 cycles of PCR at 95°C for 15 sec and 60°C for 60 sec.

### Determination of concentration of cytokines and chemokines

Cytokines and chemokines in culture fluid were measured by a multiplex assay (Bio-Plex, Bio-Rad, Hercules, CA, USA) after centrifugation of culture fluid harvested from A549 cell cultures. Human RANTES Immunoassay ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA) and a IL-12p40 minikit (BD OptEIA) was used.

### Cell proliferation assay for survival rate

A MTT assay kit (Roche, Mannheim, Germany) was used for survival rate calculation. MTT labeling reagent (10 $\mu$L of 0.5 mg/mL) provided in the kit was added to the cultured cells in a 96-well microplate with flat-bottom wells in a total volume of 100 $\mu$L, and then incubated for 24 hr at 37°C in a 5% $CO_2$ incubator. Solubilization solution (100 $\mu$L) was added to each well and the spectrophotometric absorbance of the samples was measured with a microplate reader at a wavelength of 550 nm.

### Statistical analysis

Analysis of variance was used to evaluate the statistical significance of the data in the quantitative analysis of cytokines and chemokines, using Student's $t$-test. Differences with $P$ values $> 0.05$ were considered significant.

## RESULTS

### Survival of A549 cells during influenza virus infection

When human A549 cells were infected with PR-8 influenza virus at 1000 pfu viral NS1 gene was expressed at 2 days post-infection, and its degree of expression was reduced at 4 days post-infection (Fig. 1a). The survival rate of the infected cells was not significantly different from that of uninfected cells (Fig. 1b). No morphological differences between infected and uninfected cells were observed at 2 and 4 days post-infection (Supplemental Fig. 1).

### Amounts of cytokine in A549 cells after influenza virus infection

Inflammatory cytokines such as IL-12p40, TNF-R2, TNF-$\alpha$, IL-6, IL-12p70, and IL-6 increase in the plasma and/or NPA in patients infected by avian (A/H5N1) influenza virus (13), and IFN-$\alpha$, IFN-$\gamma$ and IL-10 in patients infected with other influenza viruses (6, 21, 22). We, therefore, measured these cytokines/chemokines in the culture fluid of cells infected with PR-8 virus *in vitro*. Production of TNF-$\alpha$ and RANTES was remarkably enhanced at 2 days after infection ($P = 0.01$ and 0.044, respectively, Fig. 2a). Expression of RANTES and IL-6 in the A549 cells was significantly promoted by the infection at 2 days ($P = 0.046$ and 0.021, respectively), but not increased at 4 days (Fig. 2b). These results indicate that mRNA expression of TNF-$\alpha$, RANTES, and IL-6 was predominantly produced from the A549 cells as a result of influenza virus infection. Other cytokines and chemokines, such as type I and II IFNs and IL-12p40, were slightly promoted, whereas mRNA expression of IL-1$\beta$, IL-5, IL-8, IL-12p35, IL-6R, TNF-R2, MCP-1, M-CSF, and GM-CSF was not up-regulated by the infection (Fig. 2c). We also measured amounts of cytokine and chemokine in the culture fluid of the infected cells. IL-6, IL-8, MCP-1, and MIP-1$\beta$ were slightly induced, but there was no difference in the amounts of IL-1$\beta$, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, and IFN-$\gamma$ (Supplemental Fig. 2).

### Knockdown of transcripts of interferon-$\alpha$ and -$\beta$, tumor necrosis factor-$\alpha$, interleukin-1$\beta$, -6, and -5, regulated upon activation normal T-cell expressed and secreted and interleukin12p40 with small interfering RNA

To investigate further the system by which release of inflammatory mediators such as cytokines and chemokines is amplified in associated with infection, knockdown procedures using siRNA were applied. siTNF-$\alpha$ significantly

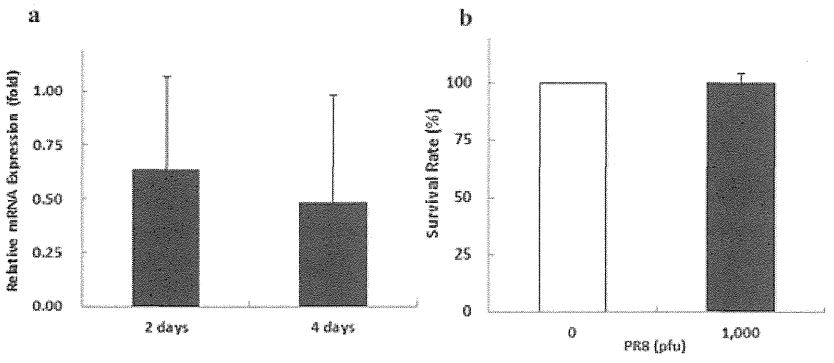


**Fig. 1. The survival rate of A549 cells during infection with PR-8.** (a) Expression of viral NS1 gene in A549 cells infected with PR-8. (b) Survival rates of infected (black bar) and uninfected (white bar) cells.

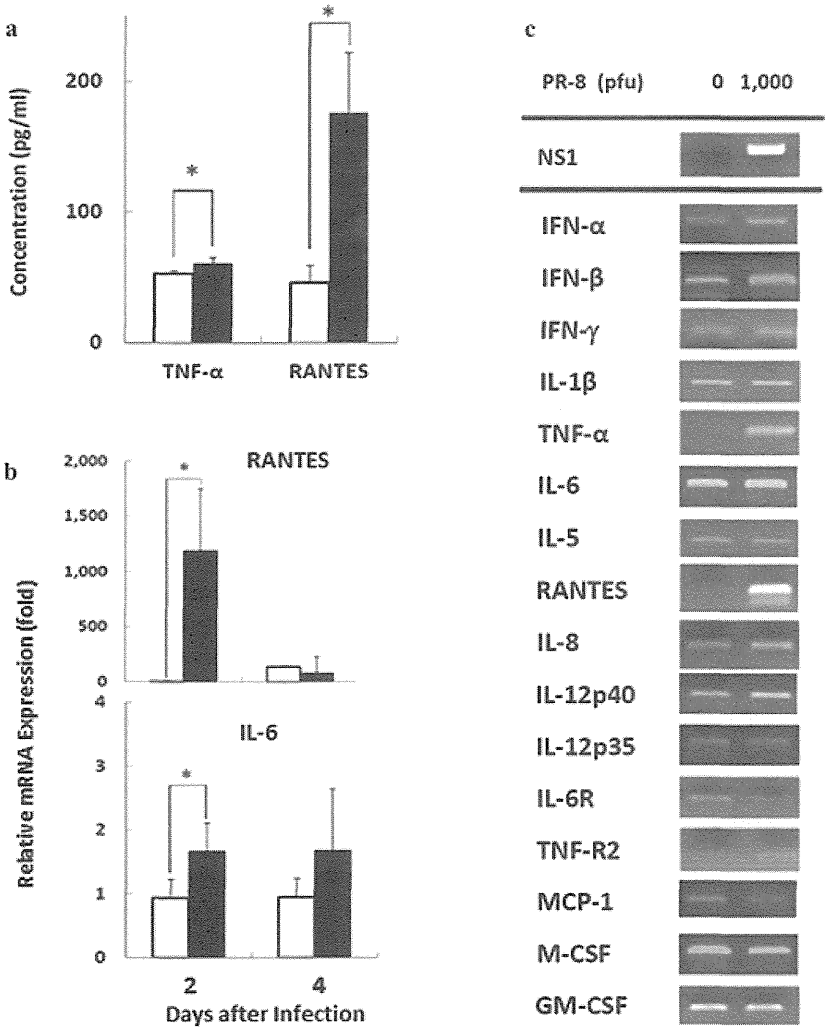


**Fig. 2. Protein production and gene expression of cytokines and chemokines in A549 cells after infection.** (a) Concentrations of TNF-$\alpha$ and RANTES secreted by infected (black bars) and uninfected (white bars) cells. (b) Quantitative analyses of expression of IL-6 and RANTES genes in infected (black bars) and uninfected (white bars) cells with PR-8 at 2 days post-infection. (c) mRNA expression of cytokine and chemokine genes at 2 days post-infection. Data are shown as mean ± SD of results from three individuals. *$P < 0.05$ (Student's $t$-test).

down-regulated the expression of RANTES ($P = 0.012$) and slightly blocked IFN-$\gamma$ expression in the infected-cells (Supplemental Fig. 3), but did not suppress expression of the other target genes, IFN-$\beta$, TNF-$\alpha$, IL-6, and IL-8. siTNF-$\alpha$ also slightly suppressed expression of TNF-$\alpha$, IFN-$\gamma$ and MIP-1$\beta$ in the infected cells at 4 days. Furthermore, siRANTES suppressed expression of target gene IFN-$\gamma$ in the infected cells at 4 days post-infection ($P = 0.033$) (Supplemental Fig. 3). However, siIFN-$\alpha$, siIFN-$\beta$, siIL-1$\beta$, siIL-6, and siIL-12p40 did not suppress

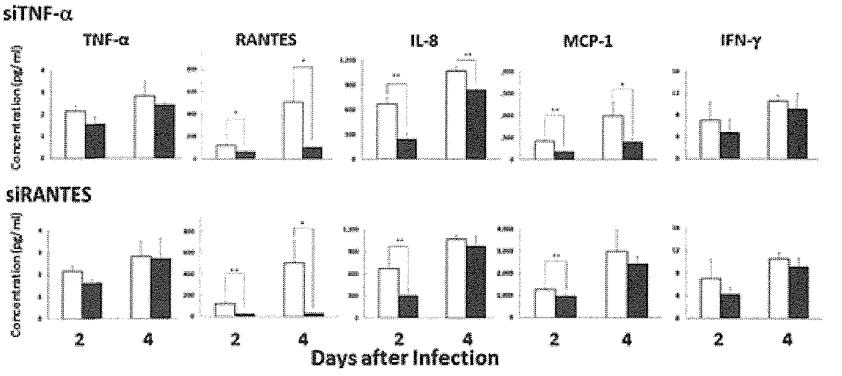


**Fig. 3. Knockdown of expression of TNF-α and RANTES genes.** Blockade by siTNF-α and siRANTES of cytokine secretion by A549 cells transfected with si-TNF-α or siRANTES (black bars) or transfected with siGAPDH as controls (white bars). Data are shown as mean ± SD of results from three individuals. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test).

the target genes IFN-α, IFN-β, IFN-γ, TNF-α, RANTES, IL-6, IL-8, and GM-CSF (data not shown).

As shown in Figure 3, siTNF-α suppressed secretion of RANTES, IL-8, and MCP-1 into the culture fluid of the infected cells at 2 days post-infection ($P = 0.01$, 0.002, and 0.00001, respectively). In addition, the siTNF-α significantly reduced the amount of RANTES, IL-8, and MCP-1 at 4 days ($P = 0.03$, 0.003, and 0.03, respectively). Moreover, siRANTES reduced secretion of RANTES, IL-8, and MCP-1 into the culture fluid of the infected cells at 2 days ($P = 0.0005$, 0.006, and 0.004, respectively), and then suppressed the amount of RANTES ($P = 0.015$) in the culture fluid at 4 days (Fig. 3). It also slightly reduced the amounts of IL-8, MCP-1, MIP-1β, and IFN-γ, but not of TNF-α (Fig. 3). We did not find any differences in amounts of other cytokines and chemokines, such as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, G-CSF, and GM-CSF, in the culture fluid of the cells at 2 and 4 days post-infection. These results suggest that TNF-α stimulates production of RANTES, IL-8, MCP-1, and IFN-γ, and RANTES enhances production of IL-8, MCP-1, and IFN-γ, but not of TNF-α, in A549 cells during influenza virus infection.

## Effect of r tumor necrosis factor-α and r regulated upon activation normal T-cell expressed and secreted on cytokine production of uninfected A549

To examine the relationship between TNF-α and RANTES in infection, we mimicked an inflammatory process in A549 cells using rTNF-α and rRANTES. Addition of rTNF-α to the culture fluid markedly increased the secretion of RANTES, IL-8, and MCP-1 from uninfected A549 cells ($P = 0.000007$, 0.00005, and 0.0000003, respectively), and slightly increased TNF-α and IL-6 production in the cells (Fig. 4). Moreover, administration of rRANTES also markedly enhanced IL-6, IL-8 and MCP-1 production ($P = 0.02$, 0.000006, and 0.000003, respectively) in the culture fluid of the A549 cells (Fig. 4). These results provide evidence that in the inflammatory process soluble TNF-α protein first stimulates RANTES production, and then the released RANTES induces production of large amounts of IL-8 and MCP-1.

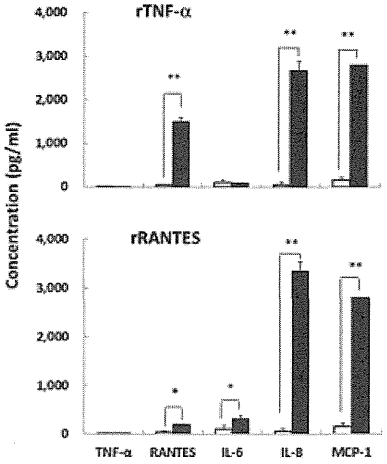


**Fig. 4. Stimulation of uninfected-A549 cells by rTNF-α or rRANTES protein.** Addition of rTNF-α or rRANTES protein (black bars), and medium (white bars). Data are shown as mean ± SD of results from three individuals. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test).

## Inhibitory role of viral nonstructural protein 1 in cells damaged by $H_2O_2$-myeloperoxidase

Myeloperoxidase is a multifunctional enzyme with unique abilities that is involved in both host defense and tissue damage at inflammatory sites. MPO activity is increased in the plasma of patients infected with avian influenza viruses (13). A recent report of ARDS induced by avian influenza virus infection A suggested that there is a relationship between MPO and IL-12 (13). We therefore analyzed the role of MPO in cell damage during influenza virus infection. In addition, because it is known to be a key factor influencing the severity of inflammation and epithelial damage, we

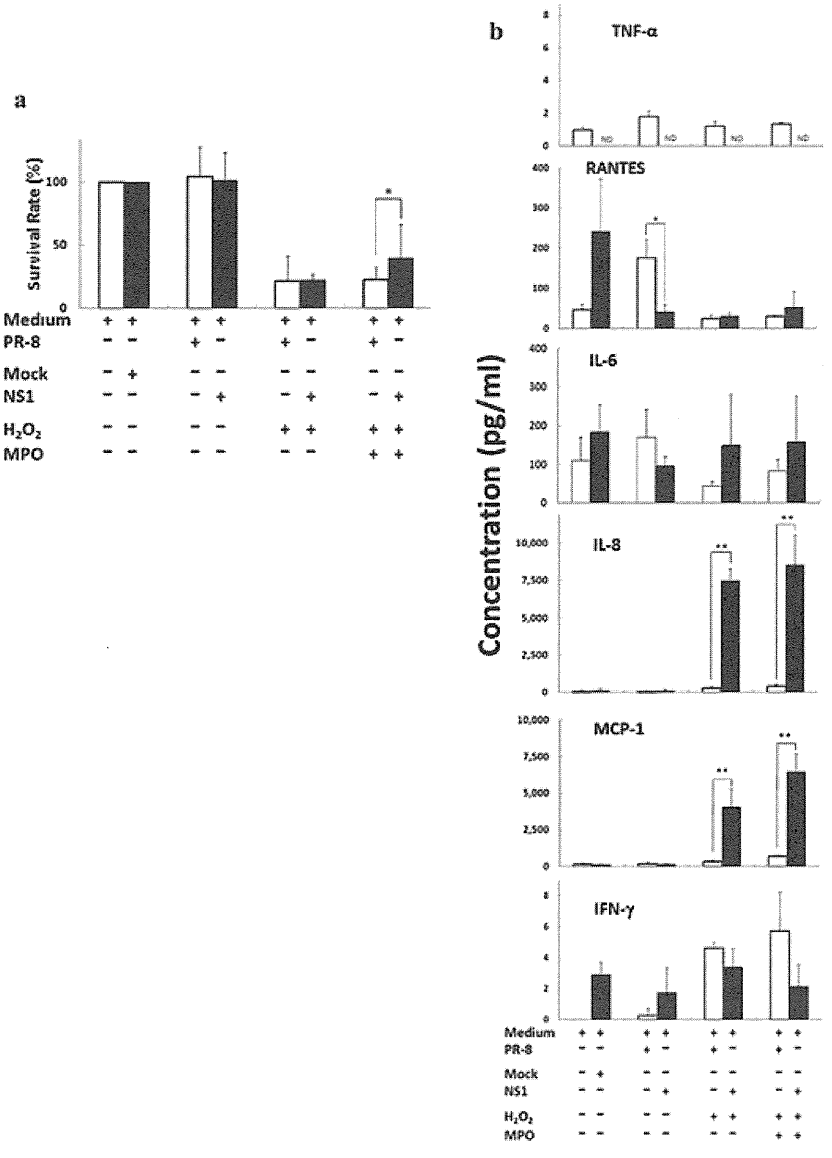


**Fig. 5. Explosion of IL-8 and MCP-1 secretion by NS1 of influenza virus collaborating with the $H_2O_2$-MPO axis.** (a) The influence of $H_2O_2$-MPO on the survival rate of A549 cells infected with PR-8 (white bars) or NS1 (black bars). (b) Cytokine production of A549 cells infected with NS1 plasmid (black bars) or PR-8 (white bars) in the presence of $H_2O_2$ and MPO. Data are shown as mean ± SD of results from three individuals. $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test).

also examined the role of NS1 of influenza virus in an *in vitro* system. First, microscopic observations showed that almost all A549 cells were damaged at 2 days after infection (Supplemental Fig. 4). In contrast, cells transfected with NS1 plasmid showed survival advantages compared to PR-8-infected cells (Fig. 5a). In addition, administration of $H_2O_2$ and MPO under uninfected or infected conditions did not result in significant differences in damage and survival rate (Fig. 5a). However, cell damage caused by $H_2O_2$ and MPO administration (3 units/mL) was significantly diminished by transfection with NS1 plasmid ($P = 0.049$, Fig. 5a), indicating that NS1 significantly protects against this type of cell damage compared with that caused by PR-8 infection. We next quantitated the concentrations of cytokines and chemokines in the culture fluid of cells that had been subjected to PR-8-infection or NS1 plasmid transfection in the presence or absence of $H_2O_2$ and MPO (3 units/mL). The increased release of RANTES from cells infected with PR-8 was suppressed by NS1 transfection (Fig. 5b). In addition, production of RANTES was decreased by the addition of MPO to the PR-8-infected cells (Fig. 5b white bar), but there was no change in concentrations of IL-8 and MCP-1. Surprisingly, addition of MPO to cells transfected with NS1 markedly enhanced concentrations of IL-8 and MCP-1 compared with PR-8 infection, but did not change the amounts of RANTES and IL-6 produced (Fig. 5b). These results indicate that NS1 produces a large amount of the chemokines

IL-8 and MCP-1 in association with the $H_2O_2$-MPO system.

## DISCUSSION

H5N1 viruses mainly infect type II alveolar epithelial cells, causing damage to lung tissue. Histopathology of samples from patients shows diffuse alveolar damage, reflecting how severely this infection damages the respiratory system (23). H5N1 infection can rapidly proceed to serious respiratory failure with a poor lower survival rate (fulminant ARDS) (4). In addition, the concentrations of cytokines and chemokines, such as IL-12p40, TNF-R2, TNF-$\alpha$, IL-6, IL-12p70, and IL-6R, are greatly increased in the plasma and NPA of ARDS patients with H5N1 infection (13). Other types of influenza viruses besides H5N1 can induce INF-$\alpha$, INF-$\gamma$, and IL-10 (21, 22). H1N1 (PR-8) virus infection targets epithelial cells in mice, causing severe ARDS.

In the present study, concentrations of TNF-$\alpha$ and RANTES had increased significantly by 2 days after PR-8 infection. On the other hand, mRNA of TNF-$\alpha$ and RANTES was strongly increased in PR-8-infected cells and that of IFN-$\alpha$, IFN-$\beta$, IFN-$\gamma$, and IL-12p40 slightly increased. These results confirm that influenza virus infection can induce expression of RANTES by normal human bronchial and nasal epithelial cells (24) and that H5N1 is more potent in inducing IP-10, TNF-$\alpha$, and RANTES (25). IFN-$\alpha$ and TNF- $\alpha$ have a significant role in priming epithelial cells to produce more cytokines and chemokines in influenza A virus infection (9). In addition, influenza A viruses induce IL-6, IL-8, and RANTES secretion from transformed bronchial epithelial cell lines *in vitro* (26), and the amounts of cytokines IL-6, TNF-$\alpha$, IFN-$\gamma$, IL-10, MCP-1, and MIP-1$\alpha$ and MIP-1$\beta$ increase in nasal lavage fluid in influenza A infection (27). On the other hand, in experiments *in vitro*, large amounts of IL-6 and IL-8 are released by normal human bronchial epithelial cells and by a human alveolar epithelial cell line treated with swine dust, which is a strong stimulus for IL-8 production by both bronchial epithelial cells and human alveolar macrophages (28, 29). These results strongly suggest that primary influenza A virus infection results in cytokine and chemokine production in human epithelial cells and that production of these factors may have a serious impact on the establishment of inflammation and virus-specific immune responses. Cytokines TNF-$\alpha$ and RANTES were being secreted from human epithelial cells A549 by 2 days after infection in the present *in vitro* study, suggesting that, in addition to the abundant cytokine secretion by infected macrophages in the alveolar region, TNF-$\alpha$ and RANTES from epithelial cells play a key role in creating "cytokine storm". Furthermore, virus-infected A549 cells express mRNA for the cytokines and chemokines TNF-$\alpha$, RANTES, IFN-$\alpha$, IFN-$\beta$, IFN-$\gamma$, and IL-12p40. In response to influenza virus infection, airway epithelial cells may express TNF-$\alpha$, RANTES, and IFNs.

When influenza A virus infects epithelial cells, it replicates in them. Macrophages and leukocytes respond to this infection by producing chemokines and proinflammatory and other immunoregulatory cytokines, leading to inflammation and alveolar damage. siRNA-induced knockdown of strong expression of inflammatory cytokines (30) clarifies the potential for induction of inflammatory cytokines and interferon responses to influenza infection (31). siRNA provides specific and robust gene silencing that can improve our understanding of gene expression and production of cytokines and chemokines. In the present study, siTNF-$\alpha$ suppressed increased expression of RANTES and IFN-$\gamma$ in cells and secretion of RANTES, IL-8, and MCP-1 by cells infected with PR-8. Moreover, siRANTES suppressed increased expression of IFN-$\gamma$ in these cells and secretion of RANTES, IL-8, and MCP-1 by them. Furthermore, in the present study administration of rTNF-$\alpha$ enhanced release of RANTES, IL-8, MCP-1, and IL-6 from uninfected A549 cells. Administration of rRANTES also enhanced the secretion of IL-6, IL-8, and MCP-1 from the cells, but not of TNF-$\alpha$. These results strongly suggest that TNF-$\alpha$ protein may be initially released from epithelial cells, then stimulate RANTES production downstream, following which released RANTES may sequentially induce the production of IL-6, IL-8, and MCP-1. Taken together, these results of studies on siRNA and administration of rTNF-$\alpha$ and rRANTES strongly suggest that production of RANTES may enhance stimulation of production of IL-6, IL-8, and MCP-1 in A549 cells infected with PR-8 virus. Therefore, TNF-$\alpha$, MCP-1, IL-8, and RANTES are primary cytokines and chemokines of cells that are associated with the pathogenesis of ARDS and may amplify the inflammatory response.

Finally, MPO, a multifunctional enzyme that is mainly found at sites of neutrophil accumulation, is involved in tissue damage at inflammatory sites in addition to its primary role in host defense. MPO activity is increased in the plasma of patients infected with influenza virus H5N1 (13). MPO not only plays an important role in the development of lung neutrophilia but also indirectly contributes to chemokine and cytokine production that may govern inflammatory processes (13, 32). In the present study, MPO provided evidence of damage to A549 cells infected with PR-8 virus. On the other hand, NS1 of type A influenza plays a key role in the regulation of production of cytokines by epithelial cells (33). However, we observed an improvement in the survival rate of A549 cells transfected with NS1 plasmid which we attributed to recovery from damage caused by the $H_2O_2$-MPO system.

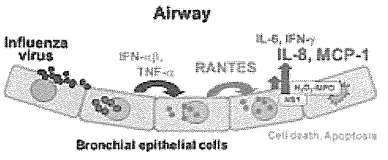


**Fig. 6. A scheme for sequential secretion and explosion of "cytokine storm" in bronchial epithelial cells infected with influenza viruses.** The first step is TNF-$\alpha$ released from bronchial epithelial cells infected with influenza virus enhances induction of RANTES secretion. In a later phase the epithelial cells associated with viral NS1 protein may enhance production of large amounts of chemokines IL-8 and MCP-1 in the presence of the $H_2O_2$-MPO system, suggesting that NS1 of PR-8 may play a key role in "cytokine storm" when the $H_2O_2$-MPO system is active.

Surprisingly, cells transfected with NS1 showed greatly enhanced concentrations of IL-8 and MCP-1 in the culture fluid. These results suggest that NS1 of PR-8 may cause production of the chemokines IL-8 by neutrophils and MCP-1 by macrophages transfected with NS1, strongly suggesting collaboration with the $H_2O_2$-MPO system in epithelial cells. On the other hand, NS1 protein is considered to be an IFN antagonist (33) and a multifunctional protein with three domains that have a number of regulatory functions during influenza virus infection (34). Therefore, explosion of IL-8 and MCP-1 induced by the NS1-$H_2O_2$-MPO system seems to be one of the roles of NS1 in epithelial cells.

The $H_2O_2$-MPO system occurs in activated neutrophils, one of the first cells to immigrate to sites of inflammation. Influenza A virus infection causes acute inflammatory disease of the lung, recruiting neutrophils and macrophages in response to chemokines IL-8 and MCP-1. When activated, these cells produce degranulation in the lung tissue due to agents such as MPO. MPO enhances mRNA expression of IL-1$\alpha$, IL-1$\beta$, and TNF-$\alpha$ (19) and may play key roles in acute lung injury, resulting in respiratory dysfunction (13). In addition, reactive oxygen intermediates produced from these cells have been shown to stimulate the production of inflammatory cytokines (19). Our study shows that NS1 and $H_2O_2$-MPO stimulate chemokine production associated with inflammatory responses. In addition, in the present study we found greatly increased concentrations of IL-8 and MCP-1 in NS1-transfected cells. This finding is similar to the observation that MPO promotes the development of lung neutrophilia and indirectly influences subsequent chemokine and cytokine production by other cell types in the lung (35). Therefore, for influenza A virus the biological activities of NS1 are likely to be potent virulence factors that implicate viral presence and inhibition of immunity. Indeed, in human virus strains and primary human cells the respective truncated NS1 polypeptides of the mutant viruses are poorly expressed and only barely detectable in overexposed blots compared to the clearly visible amounts observed in TX WT-infected A549 cells (14). In addition, Schultz-Cherry *et al.* reported that the multimerization domain of the NS1 protein, but not the effector domain, is required for apoptosis (36). However, this mutation is not sufficient to inhibit apoptosis using whole virus. In fact, the NS1 gene shows reduced pathogenesis and protection from wild-type influenza virus infection (18, 37) if the $H_2O_2$-MPO system is not present at the influenza infection site. Taken together, these observations strongly suggest that TNF-$\alpha$ regulates RANTES production, which leads to increases in IL-6, IL-8, MCP-1, and IFN concentrations. Furthermore, in the presence of the $H_2O_2$-MPO system viral NS1 protein produced in the cells is associated with enhanced production of large amounts of the chemokines IL-8 by neutrophils and MCP-1 by macrophages, suggesting that NS1 of H1N1 (PR-8) influenza virus may play a key role in "cytokine storm" when the $H_2O_2$-MPO system is active. In the initial stages of influenza viral infection, RANTES secretion, which is stimulated by TNF-$\alpha$, enhances induction of IFN-$\gamma$ in the bronchial epithelial cells. The subsequent synthesis of NS1 protein may enhance IL-8 and MCP-1 in the presence of the $H_2O_2$-MPO system produced by activated neutrophils (Fig. 6).

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## DISCLOSURE

The authors have no financial conflicts of interest.

## REFERENCES

1. Wong S.S.Y., Yuen K. (2006) Avian influenza virus infections in humans. *Chest* **129:** 156–68.
2. Cox N.J., Subbarao K. (1999) Influenza. *Lancet* **354:** 1277–82.
3. Tran T.H., Nguyen T.L., Nguyen T.D., Luong T.S., Pham P.M., Nguyen V.V.C., Pham T.S., Vo C.D., Le T.Q.M., Ngo T.T., Dao B.K., Le P.P., Nguyen T.T., Hoang T.L., Cao V.T., Le T.G., Nguyen D.T., Le H.N., Nguyen T.K.T., Le H.S., Le V.T., Dolecek C., Tran T.T., de Menno J., Schultsz C., Cheng P., Lim W., Horby P., Farrar J. (2004) Avian influenza A (H5N1) in 10 patients in Vietnam. *N Engl J Med* **350:** 1179–88.
4. Kawachi S., Luong T.S., Shigematsu M., Furuya H., Phung T.B.T., Phan H.P., Nunoi H., Nguyen T.L., Suzuki K. (2009) Risk

parameters of fulminant acute respiratory distress syndrome and avian influenza (H5N1) infection in Vietnamese children. *J Infect Dis* **200**: 510–15.
5. Suarez D.L., Schultz-Cherry S. (2000) Immunology of avian influenza virus: a review. *Dev Comp Immunol* **24:** 269–83.
6. Jong de M.D., Simmons P.C., Tran T.T., Vo M.H., Smith J.D.G., Tran N.B.C., Dang M.H., Nguyen V.V.C., Truong H.K., Vo C.D., Phan T.Q., Bach V.C., Do Q.H., Guan Y., Peiris J.S.M., Nguyen T.C., Tran T.H., Farrar J. (2006) Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* **12:** 1203–7.
7. Cheung C.Y., Poon L.L., Lau A.S., Luk W., Lau Y.L., Shortridge K.F., Gordon S., Guan Y., Peiris J.S. (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* **360:** 1831–37.
8. Chan C.W.M., Chan W.Y.R., Yu C.L.W., Ho C.C.C., Chui W.H., Lo C.K., Yuen M.K., Guan Y., Nicholls J.M., Peiris J.S.M. (2009) Influenza H5N1 virus infection of polarized human alveolar epithelial cells and lung microvascular endothelial cells. *Respir Res* **10:** 102–12.
9. Veckman V., Osterlund P., Fagerlund R., Melen K., Matikainen S., Julkunen I. (2006) TNF-$\alpha$ and IFN-$\alpha/\beta$ enhance influenza-A-virus-induced chemokine gene expression in human A549 lung epithelial cells. *Virology* **345:** 96–104.
10. Geiss G.K., Salvatore M., Tumpey T.M., Carter V.S., Wang X., Basler C.F., Taubenberger J.K, Bumgarner R.E., Palese P., Katze M.G., García-Sastre A. (2002) Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc Natl Acad Sci USA* **99:** 10736–41.
11. Beigel J.H., Farrar J., Han A.M., Hayden F.G., Hyer C.R., de Jong M.D., Lochindarat S., Nguyen T.K.T., Nguyen T.H., Tran T.H., Nicoll A, Touch S, Yuen K. (2005) Avian influenza A (H5N1) infection in humans. *New Engl J Med* **353:** 1374–85.
12. Peiris J.S., Yu W.C., Leung C.W., Cheung C.Y., Ng W.F., Nicholls J.M., Ng T.K., Chan K.H., Lai S.T., Lim W.L., Yuen K.Y., Guan Y. (2004) Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* **363:** 617–19.
13. Phung T.B.T., Luong T.S., Kawachi S., Nunoi H., Nguyen T.L., Nakayama T., Suzuki K. (2011) Interleukin 12 and myeloperoxidase (MPO) in Vietnamese children with acute respiratory distress syndrome due to Avian influenza (H5N1) infection. *J Infect* **62:** 104–08.
14. Kester H., Svetlana B., Thomas M., Adolfo G., Ana F. (2009) The NS1 protein of a human influenza virus inhibits type I interferon production and the induction of antiviral responses in primary human dendritic and respiratory epithelial cells. *J Virol* **83:** 6849–62.
15. Krug R.M., Yuan W., Noah D.L., Latham A.G. (2003) Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* **309:** 181–89.
16. Hale G.B., Jackson D., Chen Y.H, Lamb A.R., Randall E.R. (2006) Influenza A virus NS1 protein binds p85 and activates phosphatidylinositol-3-kinase signaling. *Proc Natl Acad Sci USA* **103:** 14194–99.
17. Hale G.B., Randall E.R., Ortin J., Jackson D. (2008) The multifunctional NS1 protein of influenza A viruses. *J Gen Virol* **89:** 2359–76.
18. Chuanfu Z., Yutao Y., Xiaowei Z., Xuelin L., Hongbin S., Yuxian H., Peitang H. (2010) Highly pathogenic avian influenza A virus H5N1 NS1 protein induces caspase-dependent apoptosis in human alveolar basal epithelial cells. *J Virol* **7:** 51–6.
19. Grattendick K., Stuart R., Roberts E., Lincoln J., Lefkowitz S.S., Bollen A., Moguilevsky N., Friedman H., Lefkowitz L.D. (2002) Alveolar macrophage activation by myeloperoxidase: a model for exacerbation of lung inflammation. *Am J Respir Cell Mol Biol* **26:** 716–22.
20. Ishida-Okawara A., Ito-Ihara T., Muso E., Ono T., Saiga K., Nemoto K., Suzuki K. (2004) Neutrophil contribution to the crescentic glomerulonephritis in SCG/Kj mice. *Nephrol Dial Transplant* **19:** 1708–15.
21. Kaiser L., Fritz S.R., Straus E.S., Gubareva L., Hayden G.F. (2001) Symptom pathogenesis during acute influenza: Interleukin-6 and other cytokine responses. *J Med Virol* **64:** 262–8.
22. de Maeyer E., de Maeyer-Guinard J. (1998) Interferons. In:Thomson A., ed. The Cytokine Handbook, 3rd edn. San Diego: Academic Press, pp. 491–516.
23. Nguyen T.L., Nakajima N., Le P.P., Sato Y., Hoang N.T., Pham V.H., Luong T.S., Katano H., Kumasaka T., Oka T., Kawachi S., Matsushita T., Sata T., Kudo K., Suzuki K. (2008) H5N1-infected cells in lung with diffuse alveolar damage in exudative phase from a fatal case in Vietnam. *Jpn J Infect Dis* **61:** 157–60.
24. Matsukura S., Kokubu F., Kubo H., Tomita T., Tokunaga H., Kadokura M., Yamamoto T., Kuroiwa Y., Ohno T., Suzaki H., Adachi M. (1998) Expression of RANTES by normal airway epithelial cells after influenza virus A infection. *Am J Respir Cell Mol Biol* **18**: 255–64.
25. Lam W.Y., Apple C.M.Y., Ida M.T.C., Paul K.S.C. (2010) Profiles of cytokine and chemokine gene expression in human pulmonary epithelial cells induced by human and avian influenza viruses. *J Virol* **7:** 344–53.
26. Adachi M., Matsukura S., Tokunaga H., Kokubu F. (1997) Expression of cytokines on human bronchial epithelial cells induced by influenza virus A. *Int Arch Allergy Immunol* **113**: 307–11.
27. Fritz R.S., Hayden F.G., Calfee D.P., Cass L.M.R., Peng A.W., Alvord W.G., Strober W., Straus S.E. (1999) Nasal cytokine and chemokine responses in experimental influenza A virus infection: results of a placebo-controlled trial of intravenous zanamivir treatment. *J Infect Dis* **180**: 586–93.
28. Palmberg L., Larsson B.M., Malmberg P., Larsson K. (1998) Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells. *Thorax* **53:** 260–64.
29. Wang Z., Malmberg P., Ek A., Larsson K., Palmberg L. (1999) Swine dust induces cytokine secretion from human epithelial cells and alveolar macrophages. *Clin Exp Immunol* **115:** 6–12.
30. Robbins M., Judge A., MacLachlan I. (2009) siRNA and innate immunity. *Oligonucleotides* **19:** 89–101.
31. Barik S. (2010) siRNA for influenza therapy. *Viruses* **2:** 1448–57.
32. Haegens A., Heeringa P., van Suylen R.J., Steele C., Aratani Y., O'Donoghue R.J.J., Mutsaers S.E., Mossman B.T., Wouters E.F.M., Vernooy J.H.J. (2009) Myeloperoxidase deficiency attenuates lipopolysaccharide-induced acute lung inflammation and subsequent cytokine and chemokine production. *J Immunol* **182:** 7990–6.
33. Newby C.M., Sabin L., Pekosz A. (2007) 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* **81:** 9469–80.
34. Noah D.L., Twu K.Y., Krug R.M. (2003) 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 39 end processing of cellular pre-mRNAS. *Virology* **307:** 386–95.
35. Krug R.M., Yuan W., Noah D.L., Latham A.G. (2003) Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* **309**: 181–9.

36. Schultz-Cherry S., Dybdahl-Sissoko N., Neumann G., Kawaoka Y., Hinshaw V.S. (2001) Influenza virus ns1 protein induces apoptosis in cultured cells. *J Virol* **75:** 7875–81.
37. Hyland L., Webby R., Sandbulte M.R., Clarke B., Hou S. (2006) Influenza virus NS1 protein protects against lymphohematopoietic pathogenesis in an in vivo mouse model. *Virology* **349:** 156–63.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

**Supplemental Fig. 1. Morphology of A549 cells during infection with PR-8.** Morphological observations of infected-A549 cells 2 and 4 days after infection. Bar in micrograph: 100 $\mu$m.

**Supplemental Fig. 2. Protein production and gene expression of cytokines and chemokines in A549 cells after infection.** Quantitation of protein production of cytokines and chemokines. Data are shown as mean ± SD of results from three individuals. $^{*}P < 0.05$ (Student's $t$-test).

**Supplemental Fig. 3. Knockdown of expression of TNF-$\alpha$ and RANTES genes.** Attenuated expression of TNF-$\alpha$, RANTES, and IFN-$\gamma$ genes by knockdown system with siTNF-$\alpha$ and siRANTES. Black bars, A549 cells transfected with siTNF-$\alpha$ or siRANTES; white bars, controls (transfected with siGAPDH). Data are shown as mean ± SD of results from three individuals. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's $t$-test).

**Supplemental Fig. 4. Morphology of A549 cells infected by PR-8 or transfected with NS1-plasmid collaborating with the $H_2O_2$-MPO axis.** 2 days post-infection. Bar in micrograph: 100 $\mu$m.

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