Inhibition of Complement Activation Alleviates Acute Lung Injury Induced by Highly Pathogenic Avian Influenza H5N1 Virus Infection

Shihui Sun1*, Guangyu Zhao1*, Chenfeng Liu1, Xiaohong Wu1, Yan Guo1, Hong Yu1, Hongbin Song2, Lanying Du3, Shibo Jiang3,4, Renfeng Guo5, Stephen Tomlinson6, and Yusen Zhou1

1State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China; 2Institute of Disease Control and Prevention, Academy of Military Medical Sciences, Beijing, China; 3Lindsey F. Kimball Research Institute, New York Blood Center, New York, New York; 4Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Virology, Fudan University, Shanghai, China; 5InflaRx GmbH, Jena, Germany; and 6Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina

The acute lung injury (ALI) that occurs after the highly pathogenic avian influenza H5N1 virus infection is associated with an abnormal host innate immune response. Because the complement system plays a central role in innate immunity and because aberrant complement activation is associated with a variety of autoimmune and inflammatory diseases, we investigated the complement involvement in the pathogenesis of ALI induced by H5N1 virus infection. We showed that ALI in H5N1-infected mice was caused by excessive complement activation, as demonstrated by deposition of C3, C5b-9, and mannose-binding lectin (MBL)-C in lung tissue, and by up-regulation of MBL-associated serine protease-2 and the complement receptors C3aR and C5aR. Treatment of H5N1-infected mice with a C3aR antagonist led to significantly reduced inflammation in lungs, alleviating ALI. Furthermore, complement inhibition with an anti-C5a antibody or complement depletion with cobra venom factor after H5N1 challenge resulted in a similar level of protection to that seen in C3aR antagonist–treated mice. These results indicate that excessive complement activation plays an important role in mediating H5N1-induced ALI and that inhibition of complement may be an effective clinical intervention and adjunctive treatment for H5N1-induced ALI.

Keywords: acute lung injury; H5N1 virus; complement; inflammation; complement inhibition

The highly pathogenic avian influenza (HPAI) H5N1 virus has been a public health concern since it emerged in 1997. More than 608 human cases have been reported worldwide, with a mortality rate of approximately 60% (1). Clinically, patients infected by H5N1 virus present with severe pneumonia and acute lung injury (ALI) or acute respiratory distress syndrome. Histopathological changes in the lung are highly similar to those of patients with severe acute respiratory syndrome (SARS) (2). Accumulating evidence suggests that complement is activated during H5N1 coronavirus (SARS-CoV) infection and that the progression of severe pneumonia and acute respiratory distress syndrome in patients with SARS is strongly associated with the activation of complement. Levels of serum C-reactive protein and the complement protein C4 are significantly higher in patients with SARS (3). A fragment of C3c is present in the sera of patients with SARS, and it is a strong indicator of disease severity (4). It has also been shown that SARS-CoV can directly activate complement via the lectin pathway (5). In addition, patients with SARS develop autoantibodies against human epithelial cells and endothelial cells, and these antibodies can mediate complement-dependent cytotoxicity (6). Together, these findings strongly suggest a possible pathogenic link between severe pulmonary injury and complement activation in H5N1 virus–infected patients.

The complement system is an important component of innate and adaptive immunity. However, aberrant complement activation contributes to the pathogenesis of many inflammatory and immunological diseases (7). Central effector molecules in complement-dependent inflammation include the complement-derived peptides C3a and C5a, which promote an inflammatory response by binding to their receptors C3aR and C5aR (8). C3a and C5a are chemoattractants that recruit and activate immune effector cells, and they have well documented roles in lung inflammation. C3a and C5a can increase vascular permeability, recruit and activate leukocytes, activate endothelial cells, up-regulate adhesion molecule and cytokine expression, and induce goblet cell secretion of mucus (9, 10).

To investigate the role of complement activation in H5N1 virus infection–induced ALI, we analyzed the levels of complement activation in immunocompetent mice challenged with the H5N1 virus and the effect of complement inhibition on viral infection and lung inflammation. We showed that excessive complement activation contributes to the pathogenic outcome of H5N1 virus infection and that inhibition of certain components of the complement system could significantly attenuate ALI induced by H5N1 infection, suggesting that application of complement inhibitors, in combination with antiviral treatment, represents an effective clinical intervention for the treatment of H5N1 infection.

MATERIALS AND METHODS

Mice and Viruses
Six-week-old, wild-type, female C57BL/6 mice (Laboratory Animal Center, Chinese Academy of Medical Sciences, Beijing, China) were maintained in a pathogen-free facility and housed in cages containing sterilized feed and drinking water. HPAI H5N1 virus A/Vietnam/1194/2004 (VN/1194) was grown and titered in embryonating hens’ eggs. A 50% lethal dose of virus was determined in mice after serial dilutions of the virus stock. All infectious experiments related to the H5N1 virus were performed in an approved biosafety level 3 facility. All procedures involving animals were approved by the Institutional Animal Care and Use Committees of the Laboratory Animal Center, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology.
Experiment Design

Mice were challenged intranasally with 10 50% lethal doses of H5N1 virus and divided into four groups: group 1 was treated intravenously with C3aR antagonist (1 mg/kg, SB290157; Calbiochem, Darmstadt, Germany) immediately after H5N1 challenge (H5N1+C5aRa group); group 2 was treated intravenously with anti-C3a antibody (400 μg/mouse) immediately after H5N1 challenge (H5N1+C5aAb group) (prepared as previously described [11]); group 3 was treated intraperitoneally with cobra venom factor (CVF) (2.5 μg/mouse; Kunming Biogen Science and Technology Co., Ltd.) at 24 and 48 hours, respectively, before H5N1 virus challenge (H5N1+CVF group); and group 4 was treated with PBS (H5N1+PBS group). Mice (n = 8 per group) were monitored daily for clinical symptoms, and on Day 5 after challenge mice were scored as described by Toapanta and Ross (12). On Days 1 and 5 after virus challenge, serum and lung tissues from more than three mice in each group were collected for analysis of virus titers, cytokines, histopathology, and immunohistochemistry (IHC).

Assays

Virus titers in lung tissue were detected and calculated as previously described (13). Cytokines in serum were measured using a mouse ELISA kit (DAKEWE Biotech C.O., Ltd, China). Myeloperoxidase (MPO) activity in lung homogenates was measured using a MPO ELISA kit (Hycult Biotechnology, B.V., Uden, The Netherlands) (14). Immunohistochemical staining was performed following a standard protocol (15). Total RNA was isolated from formalin-fixed, paraffin-embedded tissue sections with the RNeasy FFPE kit (Qiagen, Valencia, CA) (16), and the relative expression of MASP2, C3aR, C5aR, MBL-associated serine protease-2 (MASP2), type II IFN (IFN-γ), and type I IFN receptor (IFN-α/βR) were analyzed using the 2−ΔΔCt method (17). The oligonucleotide primers used are listed in Table 1. For details, see the online supplement.

Morbidity and Lung Injury Evaluation

Mice were individually monitored and scored for symptoms daily after virus challenge as described (12). Lung tissue damage was observed by light microscopy according to conventional procedures. Semiquantitative assessment of lung injury was performed as reported previously (15).

Statistical Analysis

All analyses were performed using Graphpad Prism Program version 5.01. The significance between different time groups was analyzed by one-way ANOVA with Dunnett’s post test. Differences in viral titer and relative IFN expression between the groups at the indicated times were analyzed using a two-way ANOVA with Bonferroni post test. The significance between survival curves was analyzed by Kaplan-Meier survival analysis with a log-rank test.

RESULTS

Activation of Complement Is Associated with ALI in Mice Challenged with H5N1 Virus

To determine whether complement activation plays a role in the occurrence of ALI induced by H5N1 virus infection, we detected complement activation products and the expression of anaphylatoxin receptors in the lungs of mice challenged with H5N1 virus. Less deposition of C3 was observed at the location of bronchial epithelial cells 1 day after challenge, but later C3 deposition increased with time, especially on epithelial cells of bronchioles and on terminal bronchioles, pneumocytes, and infiltrating lymphocytes (Figures 1A–1C). Neither apparent deposition of C5b-9 nor MBL-C nor expression of C3aR and C5aR was seen in lung tissues at Day 1 after challenge. However, deposition of MBL-C and expression of C3aR and C5aR in lung tissues increased gradually in the following days until Day 5 after H5N1 infection, especially the presence of pneumocytes and infiltrating lymphocytes (Figures 1D–1O). To confirm the immunohistochemical observations, we analyzed the gene expression of C3aR, C5aR, and MASP2 in lung tissues by relative real-time quantitative PCR in samples isolated at Days 1, 3, and 5 after H5N1 virus challenge. The expression of C3aR and C5aR mRNA was increased at Day 1 after challenge, and expression levels of C5aR mRNA increased gradually in the following days (Figures 1P and 1Q). The expression of MASP2 also increased significantly at Day 1 after H5N1 infection and increased in the following days (Figure 1R). These data indicate that complement is massively activated after H5N1 infection and that, based on the deposition of MBL-C and increased expression of MASP2, complement activation is involved in the lectin pathway.

Inhibition of Complement Activation with C3aR Antagonist Results in Attenuated Lung Injury and Increased Survival Rate of Mice Challenged with H5N1 Virus

After H5N1 virus infection, patients and experimental animals exhibit severe disease and primary viral pneumonia complicated by ALI. In our previous study on paraquat-induced ALI, we demonstrated that inhibiting the interaction of C3a and C5a anaphylatoxins with their receptors ameliorated lung injury (15). We therefore investigated whether C3aR antagonist could also attenuate ALI induced by H5N1 infection. After infection, we observed gross damage and pathologic changes in lung tissue by H&E staining. At Day 1 after infection, some parenchymal expansion and low levels of neutrophil congestion were seen in the PBS group. However, mice treated with C3aR antagonist showed no changes when compared with normal healthy mice (Figures 2A and 2B). At Day 5 after H5N1 infection, lung damage was more severe in the H5N1+PBS group, with high numbers of alveolar epithelial cells showing degeneration and collapse, infiltration of inflammatory cells accompanied by large quantities of exudates, and severe edema, especially around vessels. However, in the H5N1+C3aRa group, only parenchymal expansion with minimal neutrophil congestion was observed (Figures 2C and 2D). Gross examination of lungs also showed severe hemorrhage in the H5N1+PBS group but not in the H5N1+C3aRa group on Day 5 after H5N1 infection (Figures 2E and 2F). Semiquantitative histological analysis also indicated that lung damage in the H5N1+PBS group was more severe than that of the H5N1+C3aRa group (Figure 2G).

Clinical symptoms of mice were also evaluated as previously described (12). From Day 3 after challenge, mice in the H5N1+PBS group started to present with early symptoms, becoming more severe by Day 5 when all mice presented with ruffled fur, hunched back, and reduced activity. In contrast, only two of eight mice in the H5N1+C3aRa group showed clinical symptoms. The difference of the sickness scores between the two groups on Day 5 was significant (Figure 2H).

C3aR antagonist treatment significantly increased survival rate, with 50% mortality in the H5N1+C3aRa group compared with 100% mortality in the H5N1+PBS group on Day 9 after H5N1 virus challenge, although there were similar presentations in dead mice from the inhibited groups and the control group (P < 0.01) (Figure 2I).

Inhibition of Complement Activation with C3aR Antagonist Decreases Inflammatory Response in Mice with H5N1 Virus Infection

An extensive inflammatory response is considered a reason for high mortality and morbidity in infected patients (18). Because aberrant activation of complement contributes to the pathogenesis of many inflammatory and immunological diseases (19), we investigated whether complement activation after H5N1...
infection is associated with increased inflammation. As determined by IHC staining, low numbers of infiltrating neutrophils were observed close to vessels in the H5N1 PBS and H5N1 C3aRa groups. By Day 5, however, a significantly increased neutrophil infiltration occurred, especially in parenchymal lung tissue, in the H5N1 PBS group but not in the H5N1 C3aRa group (Figures 3A–3E). MPO activity, an indicator of neutrophil infiltration, was consistent with the IHC data (Figure 3F). Serum concentrations of the inflammatory cytokines TNF-α and IL-6 were also measured. On Day 1 after H5N1 virus infection, the concentration of TNF-α was not different in the H5N1 PBS and H5N1 C3aRa groups. However, the TNF-α level was significantly lower in the H5N1 C3aRa group compared with the H5N1 PBS group on Day 5 (Figure 3G). Although IL-6 concentrations were increased after infection, no difference in the levels of this cytokine could be found between the two groups (Figure 3H). We also measured dynamic changes of relative mRNA expression of IFN-γ and IFN-α/βR by real-time RT-PCR. C3aR antagonist reduced expression of IFN-γ mRNA at Day 5 after virus infection, although no difference in expression levels was evident for the first 3 days after infection (Figure 3I). Expression of IFN-α/βR mRNA decreased after H5N1 infection, but no difference emerged between the two groups (Figure 3J). The results show that C3aR antagonist treatment attenuates inflammation and lung injury after H5N1 virus infection.

**Inhibition of Complement Activation with C3aR Antagonist Decreases Virus Load in Lung Tissues after H5N1 Virus Infection**

To determine whether C3aR blockade has an influence on virus replication, hemagglutinin (HA) antigen expression of H5N1 virus and virus titer in lung tissues was measured. On Day 1
after H5N1 infection, IHC staining showed low levels of HA on bronchiole epithelium and low levels of lymphocytes and phagocytes in the C3aR- and PBS-treated groups (Figures 4A, 4C, and 4E). On Day 5, more extensive HA staining was observed on sections from the H5N1+PBS and H5N1+C3aRa groups, and the staining was detected mainly in bronchial epithelial cells and cell debris, endothelium, and, especially, inflammatory cells and phagocytes in interstitial tissues. However, no difference in IHC staining was apparent between the groups (Figures 4D and 4F). Therefore, virus titer was also measured. Virus titer was significantly lower in lung tissues from the H5N1+C3aRa group on Day 5 compared with the H5N1+PBS group, although no significant difference was seen on Days 1 and 3 after infection (Figure 4G). These data indicate that C3aR antagonist limits replication of the H5N1 virus.

### Inhibition of Complement Activation with Anti-C5a Antibody Attenuates H5N1 Infection–Induced Lung Injury

To further investigate how aberrant complement activation is involved in lung injury after H5N1 virus infection, we determined the effect of anti-C5a Ab treatment, which had been applied in a therapeutic role in anti-C5a in inflammatory disease models (20, 21). Similar to C3aR antagonist treatment, anti-C5a Ab  

### Table 1. Primers for Quantitative PCR

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<tr>
<th>Oligonucleotide</th>
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<tr>
<td>Mouse C3aR forward</td>
<td>TCTCACTGAGGCCATCTATTTCGTT</td>
</tr>
<tr>
<td>Mouse C3aR reverse</td>
<td>ATGCGCTCTGTCAGTCTG</td>
</tr>
<tr>
<td>Mouse C5aR forward</td>
<td>CCGCTATGTGCTTCACAT</td>
</tr>
<tr>
<td>Mouse C5aR reverse</td>
<td>ACGGGCATGACTAATGGTAG</td>
</tr>
<tr>
<td>Mouse MASP2 forward</td>
<td>ATAGAAAAGGCTGCAAGC</td>
</tr>
<tr>
<td>Mouse MASP2 reverse</td>
<td>TCCCAGAGACACTTGCAATC</td>
</tr>
<tr>
<td>Mouse IFN-γ forward</td>
<td>ATCTCCAGGAAGTCCAAGA</td>
</tr>
<tr>
<td>Mouse IFN-γ reverse</td>
<td>TTCAAGACCTCAAAAGTCTAGGA</td>
</tr>
<tr>
<td>Mouse IFN-α/β forward</td>
<td>GGGACTACATGGCTGC</td>
</tr>
<tr>
<td>Mouse IFN-α/β reverse</td>
<td>GTCTCAAGACTGCTATTTG</td>
</tr>
<tr>
<td>Mouse GAPDH forward</td>
<td>TGGGTGTAACACAGGAA</td>
</tr>
<tr>
<td>Mouse GAPDH reverse</td>
<td>AAGGCTTTTACAGG</td>
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*Definition of abbreviation: GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Figure 2. Complement inhibition with C3aR antagonist attenuated lung injury after H5N1 challenge. Wild-type BALB/c mice were challenged with H5N1 virus and then injected intravenously with C3aR antagonist (H5N1+C3aRa group) or PBS (H5N1+PBS group), and lung tissue and serum samples were analyzed at the indicated times. (A–D) Histological examination of lung tissue of mice at Days 1 and 5 after challenge. (E and F) Gross appearance of mouse lung at Day 5 after challenge. (G) Semiquantitative assessment of acute lung injury in mice at Days 3 and 5 after challenge (n = 3). (H) Sickness score of mice at Day 5 after challenge (n = 8). (I) An additional eight mice in each group were monitored until the days on which all the mice died in any of the groups. *P < 0.05. **P < 0.01. ***P < 0.001.
treatment also reduced lung injury and neutrophil infiltration especially on Day 5 after H5N1 virus infection (Figures 5A–5G). Also, anti-C5a Ab treatment increased survival rate, with 50% mortality in the H5N1+C5aAb group compared with 100% mortality in the H5N1+PBS group on Day 9 after H5N1 virus challenge \( (P < 0.001) \) (Figure 5H).

**Inhibition of Complement Activation with CVF-Attenuated ALI Induced by H5N1 Virus Infection**

To investigate if complement activation is involved in mediating lung inflammation and injury after virus infection, we depleted complement from mice with CVF before H5N1 virus challenge. At Day 1 after H5N1 virus challenge, lung damage and

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*Figure 3.* C3aR antagonist inhibition decreased the inflammatory response in lungs after H5N1 challenge. The experiment was set up as described in Figure 2. (A–D) Immunohistochemical staining of neutrophils in lungs at Days 1 and 5 after challenge in the H5N1+PBS (A, B) and H5N1+C3aR antagonist groups (C, D). (E) Semiquantitative assessment of neutrophil infiltration in lungs at Days 1 and 5 after challenge. (F–H) Myeloperoxidase activity, TNF-α, and IL-6 were measured by ELISA in serum at Days 1 and 5 after challenge. (I and J) Dynamic changes of the transcription level of IFN-γ mRNA and IFN-α/βR mRNA in lungs after challenge were analyzed by relative real-time quantitative PCR. ***p < 0.001.
inflammatory cell infiltration, as assessed by H&E and IHC staining, were attenuated in the H5N1+CVF group compared with the H5N1+PBS group. Attenuation of injury and inflammatory cell infiltration was also seen on Day 5 after infection in the CVF-treated group (Figures 6A–6H). Expression levels of IFN-γ mRNA and IFN-α/βR mRNA were also measured, and the expression level of IFN-γ mRNA was significantly lower in the CVF-treated group compared with the PBS-treated group on Days 1 and 5 after infection (P < 0.01) (Figure 6I). However, the dynamic changes of IFN-α/βR mRNA were similar between the two groups (Figure 6J). A lower virus titer on Day 5 and a higher survival rate were also observed in CVF-treated mice compared with PBS-treated mice (Figures 6K and 6L). These data further indicate an important role for complement in lung inflammation and injury after H5N1 infection, suggesting that complement inhibition may be a therapeutic option.

**DISCUSSION**

The HPAI H5N1 virus is characterized by severe ALI with presentations of pneumocyte damage, severe edema, and extensive inflammatory infiltration. A “cytokine storm” is considered the main cause of H5N1-induced ALI. Cumulative evidence suggests that although complement plays an important role in host defense against pathogens, it also contributes to tissue injury in many inflammatory disease states. Clinical data have shown that the complement activation products C3a and C5a are associated with the inflammatory response and severe lung damage that occurs after pandemic influenza virus infection (22, 23). Here, we show that HPAI H5N1 infection induces aberrant activation of complement in a mouse model and that inhibition of complement reduces lung tissue damage, decreases inflammatory cell infiltration, and reduces inflammatory cytokine levels.

**Figure 4.** C3aR antagonist decreased virus load in lung tissue. The experiment was set up as described in Figure 2. (A–F) Immunohistochemical staining of H5N1 virus antigen in lungs at Days 1 and 5 after challenge in H5N1+PBS group and H5N1+C3aR antagonist group. (G) Virus titer in lungs at Days 1, 3, and 5 after challenge in the H5N1+PBS group and H5N1+C3aR antagonist group. **P < 0.01.
Complement plays an important role in host defense and has important homeostatic functions. However, complement activation can also cause tissue injury and aberrant immune responses and is associated with various inflammatory and autoimmune diseases (19, 24, 25). As such, although C3 knockout mice have been used to study the role of complement in many inflammatory diseases (26, 27), it is not an ideal model for studying mechanisms of injury associated with infection because these mice are permanently immunocompromised and may not fully reflect the process of disease development in an immunocompetent state. Thus, in the current study, complement likely has dual roles and is a double-edged sword, protecting against infection while promoting inflammation and tissue injury. Complement can exert tissue damage via the production of multiple complement activation products such as C3a and C5a, which are important anaphylatoxins, and can degranulate mast cells and basophils, release vasoactive mediators, and induce inflammatory responses (19). Furthermore, C3a and C5a are strong chemotactants for inflammatory cells to migrate toward the site of inflammation, thus enhancing the inflammatory response (28–30). Patients with H5N1 infection present with excessive complement activation and a harmful host inflammatory response. Here, virus-induced lung injury was associated with MBL-C deposition and increased transcription levels of MASP-2 in lung tissue, suggesting that HPAI H5N1 virus infection results in activation of the MBL pathway. However, this finding does not exclude the involvement of other complement activation pathways in H5N1-driven complement activation in the host.

A “cytokine storm” is considered to be the main cause of HPAI H5N1-induced ALI in patients and in mouse models, manifesting as extremely high levels of inflammatory cytokines in sera and lung tissue (37). It was reported that the high viral
load, combined with increased levels of proinflammatory cytokines in the hosts infected by the 1918 influenza A virus, may be associated with severe disease and increased mortality (38, 39). Also, inflammatory cytokines exert a significant influence on peripheral homeostasis and injury (40, 41). Recent studies indicate a close link between increased production of proinflammatory cytokines, especially TNF-α, and disease severity and high viral loads in humans infected with the H5N1 virus. In a cohort of H5N1 virus–infected individuals, high concentrations of TNF-α in serum were accompanied with high viral loads in throat swabs (42), and the expression of TNF-α was associated with HPAI H5N1 virus in epithelial cells of alveoli in postmortem lung tissues.
(43). The complement activation products C3a and C5a not only function as chemokines but also have the capacity to induce gene expression and protein synthesis of proinflammatory cytokines such as TNF-α and IL-1β in neutrophils and macrophages (44, 45). Our study demonstrated that blockade of C3a and C5a in H5N1-infected mice significantly reduced lung inflammation and improved survival rate, indicating that complement inhibition can serve as an inflammation-limiting strategy in the treatment of ALI induced by H5N1 infection.

Type II IFN response is associated with highly pathologic H5N1 virus infection. Perrone and colleagues (46) demonstrated that a highly pathologic H5N1 virus (Thai/16) induced higher levels of cytokines levels, including IFN-γ, MIP-1α, IL-6, and KC in mouse lungs, than a low-virulence virus. IFN-γ mediates increased production of nitric oxide, which can result in enhanced recruitment of neutrophils and macrophages. Because neutrophils elicit a number of inflammatory responses in the presence of IFN-γ (47), the decrease in IFN-γ in the lungs after complement inhibition may be responsible, at least in part, for the reduced inflammatory response. Type I IFN response is a powerful host defense mechanism against H5N1 influenza virus infection, especially during early control of virus replication and spread (48, 49). However, similar IFN-α/βR transcription levels were found after C3aRa and CVF treatment when compared with a nontreatment group. This finding was confirmed by an immunohistochemistry study using a polyclonal antibody to IFN-α/βR (data not shown), suggesting that complement activation does not play an essential role in the type I IFN response.

In summary, our study has demonstrated that H5N1 virus infection results in complement activation via the activation pathways, including the MBL pathway, which leads to inflammatory response and ALI. Inhibition of complement activation could significantly decrease inflammatory responses and attenuate ALI. These data further indicate that inhibition of complement or complement activation products represents an alternative and adjunctive therapeutic option for treating ALI induced by H5N1 virus infection and that the combined treatment of antivirus and antiinflammation would be recommended.

Author disclosures are available with the text of this article at www.atijournals.org.

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