

High Level of Neutrophil Extracellular Traps Correlates With Poor Prognosis of Severe Influenza A Infection

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Background. Most patients with severe infection with influenza A virus (IAV) progress to acute respiratory distress syndrome and even multiple organ dysfunction syndrome (MODS). Neutrophil extracellular traps (NETs) can be induced by pathogens and are responsible for immune tissue damage. We conducted a prospective study on the production and effects of NETs in H7N9 and H1N1 patients.

Methods. We investigated NET production in plasma and supernatant of cultured neutrophils by measuring cell-free deoxyribonucleic acid (DNA) and myeloperoxidase (MPO)-DNA complexes with PicoGreen dye and enzyme-linked immunosorbent assay methods, respectively. We also observed NET structure by immunofluorescence staining.

Results. We found that patients with severe influenza showed elevated plasma NET level on the day of admission. Neutrophils from these patients showed higher capacity to release MPO-DNA complex in response to interleukin-8 or lipopolysaccharide stimulation. We also found that NETs from H7N9 and H1N1 patients increased the permeability of alveolar epithelial cells, and, consequently, NET production was positively correlated with acute physiology and chronic health evaluation (APACHE) II score and MODS.

Conclusions. These data indicate that high level of NETs contributes to lung injury and is correlated with severity of disease. Thus, NETs might be a key factor to predict the poor prognosis in IAV patients.

Keywords. H1N1; H7N9; interleukin-8; MODS; neutrophil extracellular traps.

Influenza is an acute viral infection circulating in humans and can cause mild to severe illness. According to World Health Organization reports, annual outbreaks of influenza result in 5%–10% infection in adults globally (<http://www.who.int/biologicals/vaccines/influenza/en/>). Among them, 3–5 million cases are estimated as severe, and 250 000–500 000 deaths occur in high-risk populations annually (<http://www.who.int/mediacentre/factsheets/2003/fs211/en/>). Although most avian influenza viruses do not infect humans, some novel virus subtypes, such as avian influenza A(H7N9) and A(H5N1), cause a potential public health threat due to higher mortality rates, lack of population immunity, and potential for efficient human-to-human transmission. From May 2009 to March 2017, 2 influenza outbreaks emerged in China, one caused by swine origin influenza A H1N1 virus in 2009, and the other caused by avian influenza A H7N9 virus starting in 2013 [1–3].

Although the frequencies of outbreak differ among influenza types, subtypes, and strains, the spectrum of illness manifestations is similar [1, 4, 5]. These patients often present with viral pneumonia, then progress to severe respiratory failure or acute respiratory distress syndrome (ARDS), which is the main cause of mortality [4, 6–9]. Thus, it is important to establish the key factors leading to lung injury. The cytokine/chemokine storm has been reported in viral pneumonia, including H7N9 and severe pandemic H1N1 infections [10–12]. Besides that, other crucial factors might also affect the outcome of influenza A virus (IAV)-infected patients.

Human neutrophils are the most abundant leukocytes with a short lifespan. Being early responder cells, neutrophils migrate to the site of infection or inflammation under the influence of several chemoattractants, such as chemokine interleukin (IL)-8 (CXCL8), CXCL10, and complement factor 5a [9, 13, 14]. Neutrophils perform their microbe-killing function by engulfing microorganisms (phagocytosis) or releasing lytic enzymes to destroy extracellular pathogens. Neutrophils have shown beneficial effects in mitigating influenza severity in a mouse model [15]. Although playing a key role in the front-line defence against invading pathogens during the acute phase of inflammation [16, 17], recent studies have shown that early and excessive infiltration of neutrophils into lungs is a common

Received 8 June 2017; editorial decision 4 September 2017; accepted 18 December 2017.

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The Journal of Infectious Diseases® 2017;XX00:1–10

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feature of pneumonia, and this correlates with lung inflammation and immunopathology [18, 19]. Pulmonary infiltration of neutrophils is associated with a fatal outcome of IAV infection [20–22]. Thus, neutrophils might be a double-edge sword in inflammation-induced tissue injury.

It has recently been reported that a variety of stimuli, including viruses, bacteria, toxic factors, and proinflammatory cytokines such as IL-8 and tumor necrosis factor- α , can trigger neutrophils to release chromatin-based structures called neutrophil extracellular traps (NETs) [23–26]. The process of NET formation is termed NETosis; a unique form of cell death that is characterized by the release of decondensed chromatin and granular contents into the extracellular space [27]. Neutrophil extracellular traps are composed of hypercitrullinated chromatin and granule proteins, such as myeloperoxidase (MPO), elastase, and cathepsin G [25]. Thus, NETs can immobilize and kill invading microorganisms [25, 28]. Neutrophil extracellular traps can be detected reliably by immunofluorescent staining of deoxyribonucleic acid (DNA) and citrullinated histone [29]. Moreover, NETs can be released into the peripheral circulatory system as cell-free DNA (cfDNA) [30], which could be quantitatively detected by a method with PicoGreen dye, an ultrasensitive fluorescent nucleic acid stain specific for double-stranded DNA.

Although NETs can trap and kill microbes, overproduction or ineffective clearance of NETs causes tissue damage by NET-associated enzymes such as MPO and elastases [23]. Studies in mice have revealed that NETs exert adverse effects in several diseases including those of the lungs. To date, NETs have been identified in mice with acute lung injury (ALI), allergic asthma, or bacterial, viral, or fungal lung infection [31–35]. In patients, platelets or human neutrophil alloantigen-3a have been identified to induce NET formation and subsequently cause transfusion-related ALI [31, 36]. In this study, by recruiting 2 independent cohorts of H7N9- and H1N1-infected patients, we revealed a close correlation between NET formation and disease severity of IAV-infected patients.

MATERIALS AND METHODS

Patients

In this prospective study, 16 patients infected with H7N9 influenza virus were enrolled at Shanghai Public Health Clinical Center in April 2013, and 11 patients with H7N9 influenza virus infection were enrolled at Beijing Ditan Hospital from April 2013 to March 2017. Sixty-six H1N1 influenza patients from November 2009 to January 2010 were collected from Beijing Ditan Hospital. Peripheral blood samples were obtained from Beijing Biobank of Clinical Resources-Emerging Infectious Disease, Biobank of Clinical Resources of Beijing Ditan Hospital, Capital Medical University. Thirty-two age- and gender-matched healthy donors were obtained as controls, who

had no infection or fever. This study was approved by the Ethics Committee of the Beijing Ditan Hospital, Capital Medical University, and Shanghai Public Health Clinical Center. Informed consent was obtained from patients or caregivers and healthy controls before enrollment. The presence of H7N9 and H1N1 influenza viruses was confirmed by reverse-transcription polymerase chain reaction. According to previous reports on epidemiology of H7N9 infection [8, 37, 38], 10 patients with H7N9 infection and 32 with H1N1 infection were diagnosed with severe illness, presenting with mild respiratory symptoms that did not have any complications throughout the clinical course, such as ARDS, multiorgan failure, and hypoxemia. Cases that met any one of the following criteria were classified as severe: presenting with severe respiratory symptoms with any complications including ARDS, shock, multiorgan failure, hypoxemia, requiring intensive care unit (ICU) admission, or mechanical ventilation for medical reasons (Table 1).

Blood Sampling and Isolation of Neutrophils

Blood samples from patients were collected within the first 3 days after hospital admission. In some of the patients, blood was taken once a week during hospitalization. Peripheral blood neutrophils were isolated by discontinuous density gradient centrifugation with Polymorphprep (Axis-Shield, Oslo, Norway). Purity of the neutrophil population was >95% and the viability was generally >90%.

Measurement of Cytokines

For serum cytokine measurement, the Cytometric Beads Array Human Inflammatory Cytokine Kit and Human Th1/Th2 Cytokine Kit (BD Biosciences, San Jose, CA) were used to determine IL-6, IL-8, and interferon (IFN)- γ . Data were acquired on a BD FACSCalibur flow cytometer.

Culture of Neutrophils

Freshly isolated neutrophils were resuspended at a final concentration of 4×10^5 cells/mL in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM glutamine supplemented with 4% heat-inactivated fetal bovine serum. The neutrophils were seeded into 24-well plates and stimulated with 100 ng/mL lipopolysaccharide ([LPS] Sigma-Aldrich, St. Louis, MO) or 100 ng/mL IL-8 (PeproTech, Rocky Hill, NJ) for 3 h at 37°C. The supernatant was obtained for cfDNA and MPO-DNA enzyme-linked immunosorbent assay (ELISA). For immunofluorescence staining assay, cells were seeded in the poly-L-lysine-coated 24-well plates before LPS or IL-8 stimulation.

Myeloperoxidase-Deoxyribonucleic Acid Enzyme-Linked Immunosorbent Assay

A capture ELISA based on MPO associated with DNA was used to quantify NET structure as previously described [36]. Values for soluble NET formation are expressed as percentage increase in absorbance above control.

Table 1. Characteristics of healthy donors and 93 patients with influenza A virus infection.

Characteristics	Healthy Donor	H7N9		H1N1	
		Mild Patients	Severe Patients	Mild Patients	Severe Patients
Patient amount	32	17	10	34	32
Age (year), mean (range)	47 (4–83)	65 (7–81)	64 (49–88)	43 (3–71)	32 (19–80)
Male sex, n (%)	19 (59.4)	11 (64.7)	9 (90)	14 (41.2)	17 (53.1)
Previous or Pre-existing Conditions, n (%)					
Respiratory diseases	0	1 (5.9)	3 (30)	0	1 (3.1)
Hypertension	5 (15.6)	8 (47.1)	3 (30)	3 (8.8)	6 (18.8)
Diabetes	3 (9.4)	3 (17.6)	2 (20)	4 (11.8)	5 (15.6)
Heart diseases	4 (12.5)	3 (17.6)	3 (30)	1 (2.9)	4 (12.5)
Autoimmune diseases	0	1 (5.9)	0	0	0
Liver diseases	0	0	1 (10)	1 (2.9)	1 (3.1)
Gestation	0	0	0	0	4 (12.5)
Positive Culture of Pathogenic Microorganism, n (%)					
Bacteria	0	1 (5.9)	4 (40)	2 (5.9)	13 (40.6)
Fungi	0	0	2 (20)	1 (2.9)	1 (3.1)
Bacteria and fungi	0	1 (5.9)	3 (30)	0	1 (3.1)
Bacterial culture of sputum	0	1 (5.9)	6 (60)	0	14 (43.8)
Bacterial culture of blood	0	0	1 (10)	0	1 (3.1)
Positive Time, n (%)					
Admission	0	0	2 (20)	0	8 (25)
Hospitalization period	0	1 (5.9)	5 (50)	0	7 (21.9)
Fever days before admission, mean (range)	0	7 (1–10)	8 (2–14)	4 (1–10)	5 (0–14)
Pneumonia before admission, n (%)	0	4 (23.5)	8 (80)	0	28 (87.5)
Antiviral treatment, n (%)	0	17 (100)	10 (100)	0	32 (100)
Antibiotics treatment, n (%)	0	4 (23.5)	10 (100)	0	32 (100)
Glucocorticoid treatment, n (%)	0	0	5 (50)	0	19 (59.4)
Immunomodulator treatment, n (%)	0	0	0	0	3 (9.4)
Platelet ($\times 10^3/\text{mm}^3$, max), mean (range)	183 (134–237)	172 (49–246)	86.3 (54–176)	139.9 (27.7–176)	136.4 (40–441)
Hemoglobin (g/L, min), mean (range)	132 (119–140)	135 (112–158)	106 (93–131)	136.6 (67–230)	115.8 (67–142)
Mechanical ventilation, n (%)	0	0	8 (80)	0	32 (100)
Clinical outcome, death, n (%)	0	0	8 (80)	0	10 (31.3)
MODS	0	0	7 (70)	0	14 (43.8)
APACHE II score, mean (range)	-	9.7 (5–22)	22.4 (7–29)	7.5 (0–14)	16.5 (5–47)
SOFA score, mean (range)	-	8.2 (8–10)	10.0 (8–15)	6.5 (4–9)	9.3 (4–15)

Abbreviations: APACHE II, acute physiology and chronic health evaluation; max, maximum; min, minimum; MODS, multiple organ dysfunction syndrome; SOFA, Sequential Organ Failure Assessment.

Measurement of Cell-Free Deoxyribonucleic Acid

The Quant-iT PicoGreen dsDNA assay was used (Invitrogen, Darmstadt, Germany) to quantify levels of cfDNA in the plasma and supernatant of neutrophils.

Detection of Neutrophil Extracellular Traps by Immunofluorescence Staining

Neutrophils were fixed, permeabilized, and blocked for immunofluorescent staining after 3-hour in vitro culture or stimulation. Cells were incubated with antibody against histone H3Cit (citrulline R2+R8+R17) and followed by secondary antibody coupled with Alexa Fluor Dyes (Invitrogen). Deoxyribonucleic acid was stained using 4',6-diamidino-2-phenylindole (DAPI), and the cells were mounted in Vectashield Mounting Media (Vector Laboratories, Burlingame, CA) for imaging with a confocal fluorescence microscope (Zeiss LSM 510 META; Carl Zeiss, Thornwood, NJ). Two independent observers manually

quantified neutrophils and NETs. Neutrophil extracellular traps were identified as structures positive for both histone H3Cit and DAPI. The percentage of NETs was calculated as an average of 5–10 fields normalized to the total number of neutrophils, and results were expressed as mean% \pm standard error of the mean (SEM).

Alveolar Epithelial Permeability Assay

The permeability assay was performed as previously reported [36]; detailed methods are provided in the Supplementary Data.

Statistical Analysis

For human data, values were presented as mean \pm standard deviation (SD). For in vitro cell experiments, values were presented as mean \pm SEM. All statistical analyses were calculated using GraphPad (San Diego, CA) Prism 6.01 software. The Kolmogorov-Smirnov test was used to inspect the normality and homogeneity

of variance of all data. *P* values were derived from the one-way Student *t* test to determine differences among several groups with normally distributed data and Mann-Whitney nonparametric test with other data. Correlations were analyzed by means of Spearman correlation analysis or Pearson correlation. For all comparisons, *P* < .05 was considered statistically significant.

RESULTS

Severe Influenza A Virus Infection Displayed Dramatically Increased Circulating Neutrophils and Serum Level of Interleukin-8

To determine whether NET formation was associated with pneumonia in H7N9 or H1N1 IAV-infected patients, 27 H7N9-infected and 66 H1N1-infected patients were enrolled. The characteristics of the patients with H7N9 or H1N1 influenza infection are summarized in [Table 1](#). Ten H7N9 and 32 H1N1 patients were diagnosed with severe pneumonia.

Compared with healthy controls and mild cases, there was a dramatic increase in the numbers and percentages of circulating neutrophils in severe cases of H7N9 and H1N1 infection at initial diagnosis ([Figure 1A](#)). We assessed serum levels of several inflammatory cytokines and found that mild and severe cases displayed comparable levels of IFN- γ . The level of IL-6 was increased in patients with severe compared with mild H7N9 infection, which indicated intensive inflammation in the former. Serum levels of IL-8, a neutrophil chemotactic factor and an inducer of NET formation, significantly increased in patients with severe H7N9 and H1N1 infection (mean \pm SD, H7N9 patients: 53.97 \pm 35.96 pg/mL vs controls: 17.06 \pm 13.56 pg/mL, *P* = .0008; H1N1 patients: 30.75 \pm 27.87 pg/mL vs 4.81 \pm 3.71 pg/mL, *P* < .0001) ([Figure 1B](#)). Serum IL-8 level was positively correlated with neutrophil counts in both H7N9 and H1N1 patients ([Figure 1C and D](#)).

Elevated Plasma Levels of Neutrophil Extracellular Traps in Patients With Severe Influenza A Virus Infection

We could not directly assess NET formation in the lungs of patients with IAV infection, thus, we measured the levels of cfDNA and MPO-DNA complex in the plasma of patients. As shown in [Supplementary Figure 1](#), 2 NET-detecting approaches were consistent with each other (*r* = 0.6020, *P* < .0001). Compared with healthy controls, patients with mild H7N9 infection showed a slight increase in cfDNA levels (mean \pm SD, 353.3 \pm 150.0 ng/mL vs 248.6 \pm 38.95 ng/mL, *P* = .0005). A more dramatic increase was detected in patients with severe H7N9 infection (mean \pm SD, 706.3 \pm 428.9 ng/mL, 95% CI 399.4–1013 ng/mL vs 353.3 \pm 150.0 ng/mL, 95% CI 276.2–430.4 ng/mL, *P* = .0032) compared with mild cases ([Figure 2A](#)). Consistent with the results of cfDNA content, a 2.4- and 6.9-fold increase in MPO-DNA levels was detected in patients with mild and severe H7N9 infection, respectively, compared with healthy controls ([Figure 2B](#)). Similar results were observed in patients with mild and severe H1N1 infection ([Figure 2A and B](#)). In both cohorts, the plasma levels of cfDNA and MPO-DNA

complex were correlated with serum IL-8 levels (H7N9 patients: cfDNA vs IL-8, *r* = 0.5177, *P* = .0057; MPO-DNA complex vs IL-8, *r* = 0.5965, *P* = .0010; H1N1 patients: cfDNA vs IL-8, *r* = 0.4609, *P* < .0001; MPO-DNA complex vs IL-8, *r* = 0.4537, *P* = .0001) ([Figure 2C and D](#)). These results indicated a higher level of NETs and IL-8 in patients with severe IAV infection.

Elevated Plasma Neutrophil Extracellular Trap Levels Correlate With Severity of Influenza A Virus Infection

We determined whether elevated plasma NET level was correlated with clinical parameters. Although patients with severe influenza showed a significant decrease in the arterial oxygen tension PaO₂/FiO₂ ratio compared with mild cases ([Supplementary Figure 2A](#)), plasma cfDNA levels were not correlated with PaO₂/FiO₂ values ([Supplementary Figure 2B](#)).

We further tested whether elevated plasma NET level was correlated with systemic inflammatory injury in the patients with IAV infection. In patients with H7N9 and H1N1 infection, plasma levels of cfDNA and MPO-DNA complex were positively correlated with acute physiology and chronic health evaluation (APACHE) II score; a classification system to estimate disease severity on the first day in the ICU ([Figure 3A and Supplementary Figure 3A](#)). We also observed higher levels of C-reactive protein (CRP) in patients with severe influenza; the level of which was also correlated with plasma cfDNA ([Supplementary Figure 2C and D](#)). These data suggest that plasma levels of cfDNA and MPO-DNA complex might be more closely correlated with systemic inflammatory injury.

Consistent with the above findings, we noticed that patients with MODS exhibited higher levels of cfDNA and MPO-DNA complex than those without MODS ([Figure 3B and Supplementary Figure 3B](#)). Higher levels of cfDNA and MPO-DNA complex were also observed in the plasma of 18 patients who died compared with 24 patients who recovered (cfDNA: recovery, 622.5 \pm 443.4 pg/mL vs died, 932.4 \pm 486.1 pg/mL, *P* = .0199; MPO-DNA complex: recovery, 3.32 \pm 2.61 pg/mL vs died, 6.14 \pm 4.13 pg/mL, *P* = .0094) ([Figure 3C and Supplementary Figure 3C](#)). Consistently, we also found that dynamic cfDNA level was higher in the patients who died than in those who recovered ([Supplementary Figure 4](#)). These data supported the notion that plasma levels of cfDNA and MPO-DNA complex were correlated with disease severity and outcome of IAV infection.

In Vitro and in Vivo NETosis After Influenza A Virus Infection

To evaluate NET formation directly, we isolated circulating neutrophils from the IAV-infected patients within 3 days of hospitalization and the healthy controls ([Figure 4A and Supplementary Figure 5A](#)), and we cultured these cells in RPMI 1640 medium without further stimulation. Three hours later, immunofluorescence staining with DAPI and H3Cit revealed a marked increase of NET formation by neutrophils from severe cases of H7N9 and H1N1 infection ([Figure 4A, B, and Supplementary Figure 5C](#)).

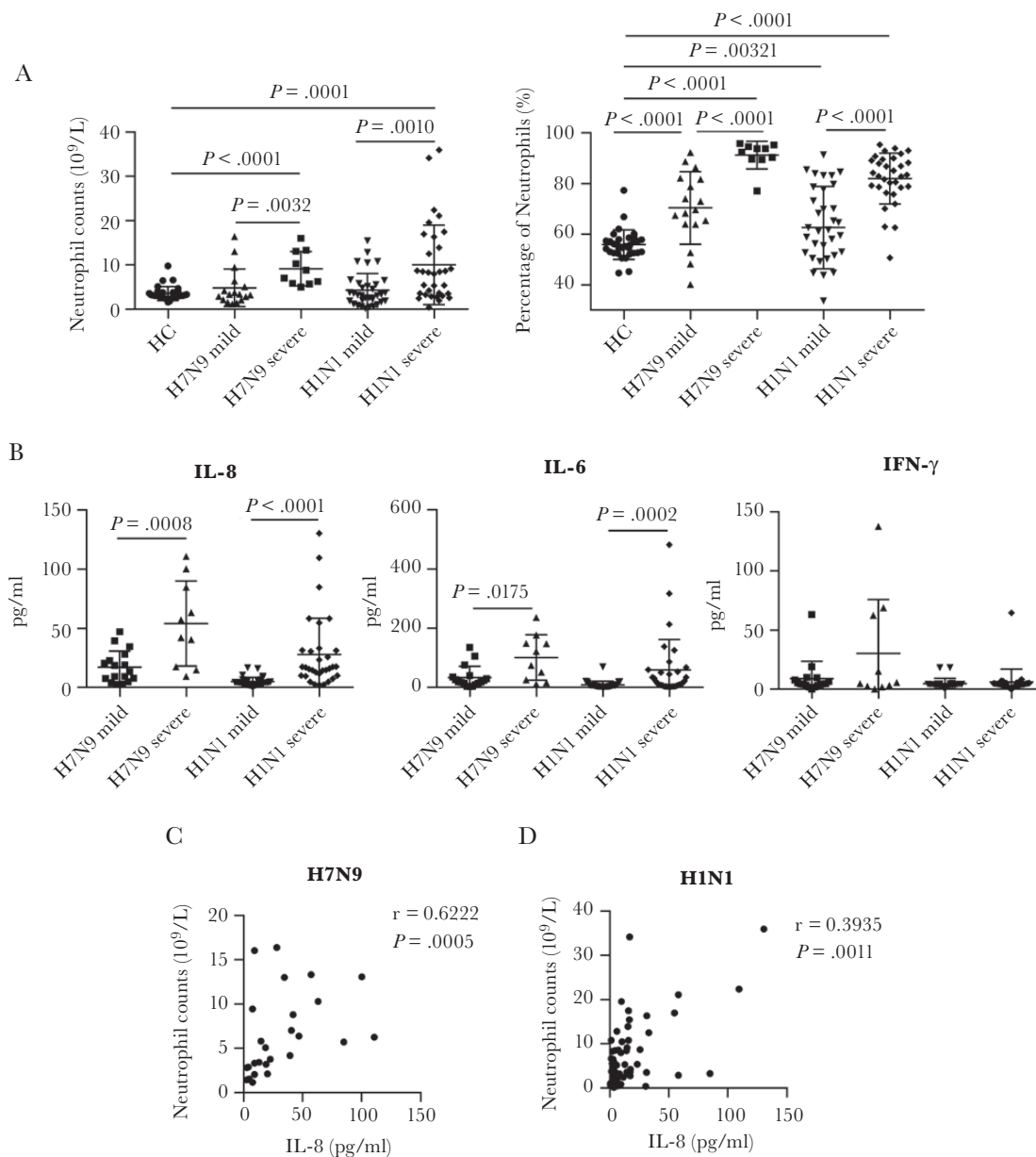


Figure 1. Levels of neutrophils and interleukin (IL)-8 were higher in severe than mild influenza. The data were obtained from patients upon hospital admission. (A) Count and proportion of neutrophils among healthy controls and the patients after different types and extents of infection. (B) Serum levels of IL-8, IL-6, and interferon (IFN)- γ in patients. Data are presented as mean \pm standard deviation. (C) Correlations between IL-8 level and neutrophil count in 27 patients with H7N9 infection. Pearson correlation analysis (r) and P values are provided in each graph. (C) Correlations between IL-8 level and neutrophil count in 66 patients with H1N1 infection. P values were obtained by unpaired t test and Mann-Whitney test. Correlations were calculated by Pearson and Spearman correlation analysis (r). Healthy control (HC), $n = 32$; H7N9 mild, $n = 17$; H7N9 severe, $n = 10$; H1N1 mild, $n = 34$; H1N1 severe, $n = 32$. Data are representative of 2 independent experiments.

To quantitate NET formation further, we measured the amount of MPO-DNA complexes in the supernatant of cultured neutrophils, and we found a significant increase in the samples from severe cases of H7N9 infection, indicating that these cells have been activated in vivo and are capable of forming NETs. Moreover, NETs released from in vitro-cultured neutrophils gradually declined with disease remission (Figure 4A and C).

Next, we determined whether the neutrophils from the patients could still form NETs in response to continuous or secondary infections. Thus, we further stimulated the neutrophils

with LPS and IL-8. As expected, the neutrophils from patients showed a greater capacity to release NETs than those from healthy controls (Figure 4D).

To confirm IAV-induced NET formation in the lungs, we infected C57BL/6 mice with PR8 (H1N1) virus. Six days post-infection, significant bronchial epithelial damage and parenchymal inflammation were observed in PR8-infected mice (Supplementary Figure 6A and B). In addition, double staining with DAPI and H3Cit revealed NET formation in the lungs. Compared with control mice, PR8-inoculated mice also

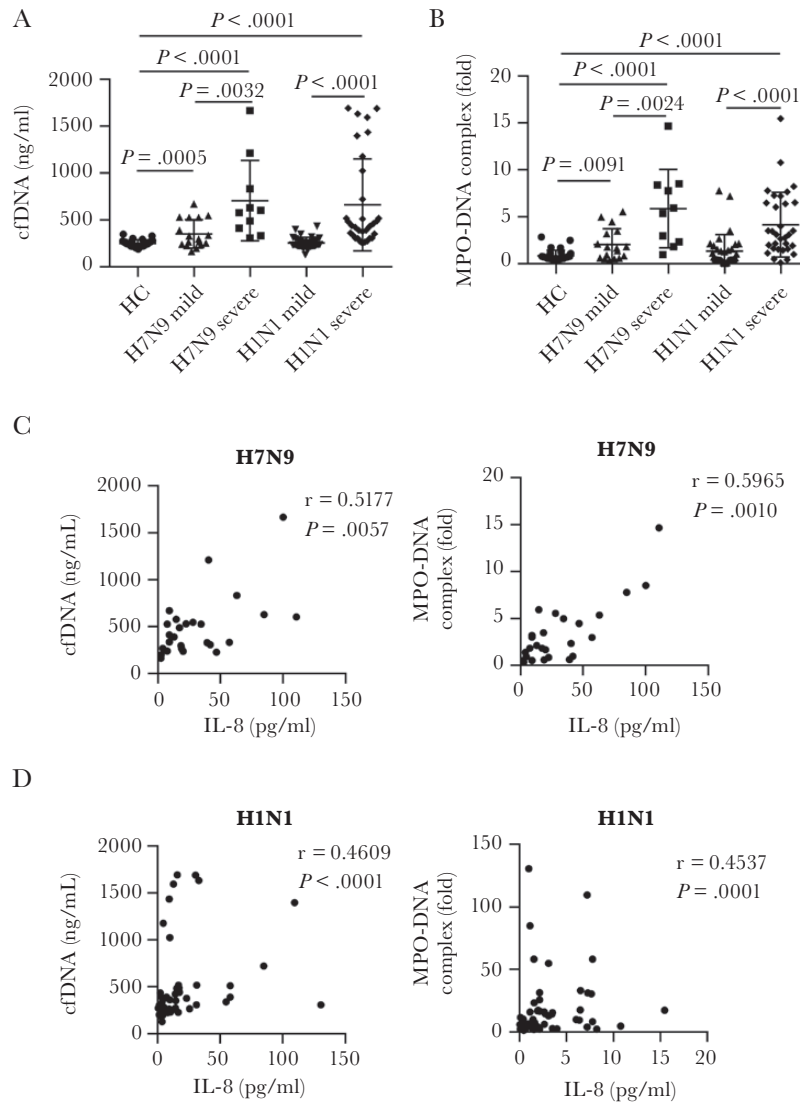


Figure 2. Plasma cell-free deoxyribonucleic acid (cfDNA) and level of myeloperoxidase (MPO)-DNA complex were increased in patients with influenza A virus infection. The plasma was obtained from patients on admission. (A) Plasma cfDNA levels were detected using the Quant-iT PicoGreen dsDNA assay. (B) MPO-DNA complex in plasma was detected with MPO-DNA enzyme-linked immunosorbent assay. Healthy control (HC), $n = 32$; H7N9 mild, $n = 17$; H7N9 severe, $n = 10$; H1N1 mild, $n = 34$; H1N1 severe, $n = 32$. Data are presented as mean \pm standard deviation. P values were obtained by unpaired t -test and Mann-Whitney test. (C) Correlations between interleukin (IL)-8 level and cfDNA, IL-8 and MPO-DNA complex level in 27 patients with H7N9 infection. (D) Correlations between IL-8 level and cfDNA, IL-8, and MPO-DNA complex in 66 patients with H1N1 infection. Spearman correlation analysis (r) and P values are provided in each graph. Data are representative of 3 independent experiments.

displayed a greater level of cfDNA in bronchoalveolar lavage and plasma (Supplementary Figure 6C and D).

Neutrophil Extracellular Traps From Influenza A Virus-Infected Patients Increase Permeability of Primed Alveolar Epithelial Cells

Increased alveolar epithelial protein permeability is a significant characteristic of IAV-induced ALI. To test whether NETs released from neutrophils of IAV patients caused lung damage, we performed a permeability assay using A549 cells in a Transwell model, as described previously [36]. As shown in Figure 5, the culture supernatant of neutrophils from H7N9-infected patients significantly increased the permeability of A549 cells. The increase in permeability was attenuated by DNase I, which

could resolve NET structure. Similar results were achieved with the plasma from patients with severe H7N9 and H1N1 infection (Supplementary Figure 7). Thus, NETs could increase permeability of alveolar epithelial cells. These results indicated that overproduction of NETs is a cause of lung injury during IAV infection.

DISCUSSION

As major causes of mortality in severe cases of IAV infection, ALI and ARDS have drawn much attention. In recent studies, NETosis has been recognized as an anti-pathogenic mechanism that contributes to pathogen control and elimination. However, NETs also exert adverse effects in several diseases by causing tissue damage [31, 36, 39]. Here, with H1N1-infected mice and

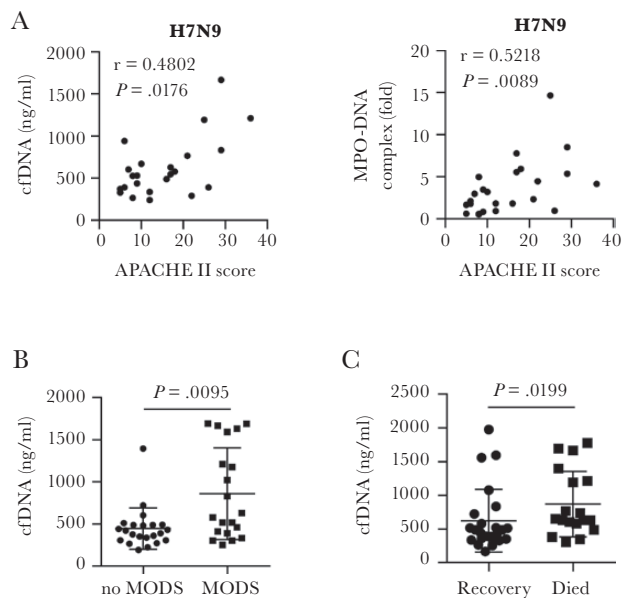


Figure 3. Plasma levels of cell-free deoxyribonucleic acid (cfDNA) and myeloperoxidase (MPO)-DNA complex were correlated with disease severity and outcome. (A) Correlations between acute physiology and chronic health evaluation (APACHE) II score and cfDNA, and level of MPO-DNA complex in 24 patients with H7N9 infection. The APACHE II scores were evaluated within the initial 24 hours of being admitted to an intensive care unit. (B) Plasma levels of cfDNA in multiple organ dysfunction syndrome (MODS) and non-MODS patients after severe H7N9 and H1N1 infection: in the non-MODS group, H7N9 (n = 3) and H1N1 (n = 18); in the MODS group, H7N9 (n = 7) and H1N1 (n = 14). (C) Plasma levels of cfDNA in severe H7N9 and H1N1 patients: recovery (n = 24) and dead group (n = 18). Pearson and Spearman correlation analysis (r) and P values are provided in each graph. Data are presented as mean \pm standard deviation. P values were obtained by Mann-Whitney test.

clinical cohorts of H7N9- and H1N1-infected patients, our data suggest that NETs play an important role in IAV-induced lung damage. The results of increased epithelial cell permeability induced by NETs confirmed the damaging effect of NETs. More importantly, we found that the plasma levels of cfDNA and MPO-DNA complex were correlated with severity of IAV infection, which indicated that these parameters might be a biomarker for progression of IAV infection.

The lungs are a direct target organ for IAV; therefore, NET formation in the lungs could directly cause lung injury, as indicated in IAV-challenged mice. Previous studies have suggested that NET components in the inflammatory tissues could be released into the bloodstream. In recent studies, cfDNA has received special attention because of its potential application as a non-invasive, rapid, and sensitive tool for molecular diagnosis. Cell-free DNA is also regarded as neutrophil-derived and is used to determine NET production in plasma [30, 34]. However, cfDNA could also be derived from other cells, such as hepatocytes, under some pathological conditions. To assess circulating NETs more specifically, we adopted an additional method to detect MPO-DNA complexes, because MPO is a specific marker for neutrophils and MPO-DNA complex is a typical structure of NETs. We

found that the results of 2 approaches for detecting NETs were consistent with each other. We also found higher levels of MPO-DNA complex in the plasma of severe than mild cases, which indicated higher levels of NET formation in severe cases.

Severe IAV infection causes ALI and can also lead to MODS. In our cohorts, 7 of 10 patients with severe H7N9 influenza and 14 of 32 with severe H1N1 influenza developed MODS. We found that circulating neutrophils from the IAV patients formed NETs in vitro, with or without further stimulation. Thus, elevated levels of circulating MPO-DNA complex were (1) attributed to NET components released from the lungs and (2) connected with systemic inflammatory responses. The patients with MODS exhibited higher levels of cfDNA and MPO-DNA complex than patients without MODS. In both H7N9 and H1N1 patients, plasma levels of cfDNA or MPO-DNA complex were not correlated with PaO₂/FiO₂ ratio but showed a strong relationship with APACHE II score. This indicates that circulating NET components might be more closely correlated with systemic inflammatory injury. This hypothesis was further supported by our findings that serum levels of CRP were also correlated with plasma cfDNA.

It has been shown that IAV-induced lung injury can trigger rapid influx of neutrophils into the lungs. Interleukin-8 is one of the major chemokines for neutrophils that is stored in alveolar macrophages, fibroblasts, microvascular endothelial cells, and airway smooth-muscle cells [40]. Interleukin-8 provides a rapid pathway for specific activation of neutrophil adhesion at sites of acute inflammation [41]. High concentrations of IL-8 in bronchoalveolar lavage fluid from ARDS patients are associated with increased neutrophil influx into the air spaces. Interleukin-8 might play a unique role in NET-induced lung injury by recruiting neutrophils from the peripheral blood to the organ of infection or inflammation, as well as by activating neutrophils to form NETs. Consistent with this notion, in our 2 cohorts, patients with severe H1N1 or H7N9 infection also exhibited higher serum levels of IL-8 than patients with mild disease. Interleukin-8 levels were also highly correlated with cfDNA levels in plasma. Thus, IL-8 might be the therapeutic target to control NET generation. It is worth noting that CXCR2 (a receptor for IL-8) antagonist danirixin is currently undergoing controlled studies and is expected to block chemotaxis of neutrophils into lungs in disease states [42]. The NET level might be affected in patients with IAV infection who received treatment with danirixin.

In addition to IL-8, Toll-like receptor (TLR) ligands can also induce abundant NET formation. Patients infected with IAV, particularly in severe cases, are always threatened by secondary infection, which is one of the most frequent causes of death after severe virus infection. In our cohorts, all of the patients with severe H7N9 infection and 46.9% of those with severe H1N1 infection developed secondary bacterial or fungal infections. We found that the neutrophils isolated from IAV patients produced NETs without activation. However, in vitro LPS stimulation further induced these

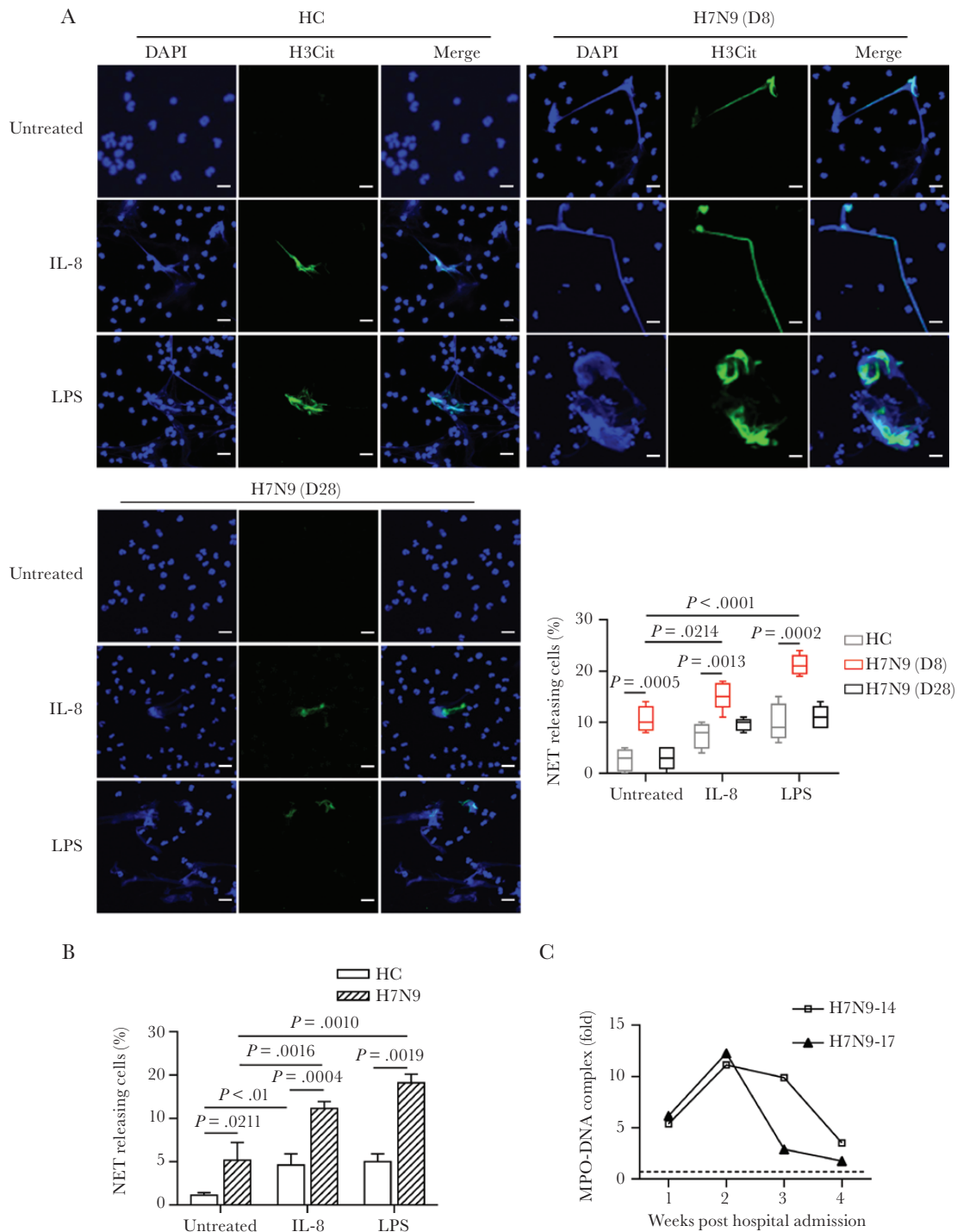


Figure 4. Neutrophil extracellular trap (NET) formation was elevated in peripheral neutrophils from patients with severe H7N9 infection, spontaneously and stimulated in vitro. (A) Immunofluorescent staining for NET formation. Neutrophils were separated from healthy controls (HC) and patients with severe H7N9 influenza on days 8 and 28 postinfection. Cells were untreated or stimulated with 100 ng/mL lipopolysaccharide (LPS) or 100 ng/mL interleukin (IL)-8 in vitro for 3 hours. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and shown as blue (DAPI), citrullinated histone H3 was detected with specific antibody and shown as green (H3Cit). The dual-labeled immunofluorescent staining is shown as a merged figure. The magnification was $\times 40$. Figure shows a representative image from 1 of 3 patients. Scale bar = 20 μm . The percentage of NETs was calculated as an average of 5–10 fields normalized to the total number of neutrophils, and results are expressed as mean \pm standard error of the mean (SEM). (B) The relative level of myeloperoxidase-deoxyribonucleic acid (MPO-DNA) complex in supernatant of neutrophils untreated or stimulated with LPS or IL-8 for 3 hours, respectively. Neutrophils were separated from HC and patients with severe H7N9 influenza on day 8 postinfection. Data are presented as mean \pm SEM. *P* values were obtained by unpaired *t* test. (C) Dynamic spontaneous NET formation of neutrophils from patients nos. 14 and 17 with severe H7N9 influenza. Neutrophils were separated from patients with severe H7N9 influenza 4 weeks postinfection. Cells were cultured for 3 hours, and the supernatants were obtained to detect MPO-DNA complexes. Data are presented as mean \pm standard deviation. The dashed line indicates the upper limit value of the HC. Data are representative of at least 3 independent experiments.

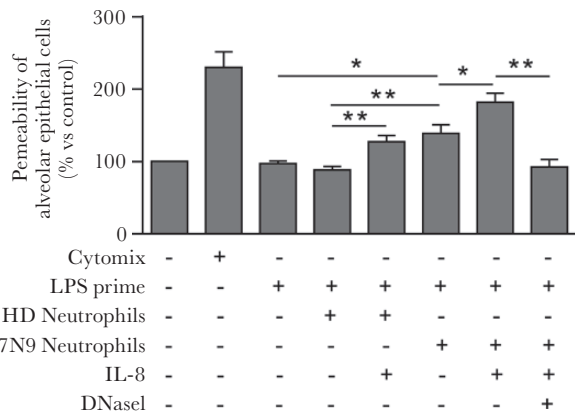


Figure 5. Permeability of alveolar epithelial cells measured in a Transwell system. (A) Supernatant containing neutrophil extracellular traps increased permeability of epithelial cells. In selected experiments, A549 cells were primed with lipopolysaccharide (LPS) 2 $\mu\text{g}/\text{mL}$ for 24 hours before the experiment. Supernatant of neutrophils stimulated with or without interleukin (IL)-8 was added to Transwells for 3 hours. In DNase I experiment, 10 U/mL DNase I was added at the same time as IL-8 stimulation. Permeability was measured by fluorescein isothiocyanate-labeled bovine serum albumin across an epithelial monolayer over 1 hour. Treating with CytoMix (tumor necrosis factor- α , IL-1 β , and interferon- γ ; 0.5 ng/mL) for 3 hours was set as a positive control. Data are presented as mean \pm standard error of the mean. *P* values were obtained by unpaired *t* test. Data are representative of 3 independent experiments.

cells to release NETs. Thus, NETs may participate in IAV-induced injury and play a role in promoting tissue damage during secondary infection. Moreover, organ damage could lead to the release of damage-associated molecular patterns, which are endogenous activators of TLRs. Therefore, on one hand, NETs components released from originally injured organs into the circulation might convert inflammatory responses into systemic responses; on the other hand, amplified inflammatory responses might lead to excessive NET formation. Such a vicious circle might be a reason for stronger inflammatory responses in severe IAV cases.

CONCLUSIONS

In conclusion, our study indicated an important role of NETs in IAV-induced inflammatory injury. Further studies are necessary to explore therapeutic regimens targeting NET formation in severe IAV infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Authors contributions. H. Ze., L. Z., and Y. Z. designed the study and analyzed and interpreted the data; H. Ze. and L. Z. drafted the article; L. Z., L. L., Y. Z., S. L., and

G. L. performed the experiments; J. X., Z. C., and X. L. collected the patients; Y. H., J. H., and B. W. handled the blood samples; A. L., J. L., L. P., and H. X. provided the clinical data; H. Zh. revised the manuscript.

Financial support. This work was funded by grants from the National Natural Science Foundation of China (No. 81570372 and No. 81501692), the Natural Science Foundation of Beijing Municipality (No. 7142080), Beijing Municipal Commission of Education of Science and Technology Project (No. KM201510025026), Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (No. ZYLX201602NHFPC), Beijing Municipal Administration of Hospitals' Youth Programme (QML20161802), and Beijing Key Laboratory of Emerging Infectious Disease Open Research Topics (No. DTKF-2016-04).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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