IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Cytokine and Chemokine Profiles in Lung Tissues from Fatal Cases of 2009 Pandemic Influenza A (H1N1)

Role of the Host Immune Response in Pathogenesis

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Pathological studies on fatal cases caused by 2009 pandemic influenza H1N1 virus (2009 pH1N1) reported
extensive diffuse alveolar damage and virus infection predominantly in the lung parenchyma. However, the
host immune response after severe 2009 pH1N1 infection is poorly understood. Herein, we investigated
viral load, the immune response, and apoptosis in lung tissues from 50 fatal cases with 2009 pH1N1 virus
infection. The results suggested that 7 of the 27 cytokines/chemokines showed remarkably high
expression, including IL-1 receptor antagonist protein, IL-6, tumor necrosis factor-α, IL-8, monocyte
chemoattractant protein-1, macrophage inflammatory protein 1-β, and interferon-inducible protein-10 in
lung tissues of 2009 pH1N1 fatal cases. Viral load, which showed the highest level on day 7 of illness onset
and persisted until day 17 of illness, was positively correlated with mRNA levels of IL-1 receptor antagonist
protein, monocyte chemoattractant protein-1, macrophage inflammatory protein 1-β, interferon-
inducible protein-10, and regulated on activation normal T-cell expressed and secreted. Apoptosis was
evident in lung tissues stained by the TUNEL assay. Decreased Fas and elevated FasL mRNA levels were
present in lung tissues, and cleaved caspase-3 was frequently seen in pneumocytes, submucosal glands,
and lymphoid tissues. The pathogenesis of the 2009 pH1N1 virus infection is associated with viral
replication and production of proinflammatory mediators. FasL and caspase-3 are involved in the pathway
of 2009 pH1N1 virus-induced apoptosis in lung tissues, and the disequilibrium between the Fas and FasL
level in lung tissues could contribute to delayed clearance of the virus and subsequent pathological
damages. *(Am J Pathol 2013, 183: 1258—1268; http://dx.doi.org/10.1016/j.ajpath.2013.06.023)*

Influenza A viruses can cause recurrent epidemics with
substantial human morbidity and mortality. The H1N1
influenza pandemic of 1918, the worst pandemic in recorded
history, killed 20 to 50 million people worldwide.[1] The
emergence of 2009 pandemic influenza A H1N1 virus (2009
pH1N1) has brought renewed concerns on the strategies for
prevention, control, treatment, and reduction of the social and
human costs of the influenza disease.[2] The 2009 pH1N1 virus
was identified as the cause of outbreaks of respiratory tract
infection, beginning in the spring of 2009, and by the time the

World Health Organization declared the pandemic over in
August 2010, the virus had spread to >214 countries, with
>18,000 deaths reported worldwide (*http://www.who.int/
csr/don/2010_08_06/en*, last accessed August 8, 2013).

The 2009 pH1N1 infection is pathologically distinct
from seasonal influenza virus H1N1 or H3N2 infection.[3]

In previous influenza pandemics, pathological evaluation of respiratory tract tissues from influenza-associated deaths suggested that marked differences exist in viral tropism and tissue damage compared with seasonal influenza virus infections.[4–8] Studies of previous pandemic deaths concluded that most (96%) of these deaths were likely due to viral infection, concurrent with secondary bacterial pneumonia.[6,9,10] A significant proportion of 2009 pH1N1-infected fatal cases showed severe pneumonia.[11] Histopathological studies of 2009 pH1N1 virus infection showed that extensive diffuse alveolar damage is the most consistent finding, and the virus predominantly infected the lung parenchyma.[3,12] In addition, fatal cases are mainly young to middle-aged patients,[13] and 40% to 60% of fatal cases have an underlying medical condition.[13–16] Previous studies suggested that inadvertent host immunological responses play an important role in the pathogenesis of H5N1[17,18] and SARS-CoV[19] infections. Recent studies suggested that the 2009 pH1N1 virus could induce higher levels of proinflammatory cytokines in the lungs of infected mice compared with mice infected with seasonal H1N1 influenza virus.[20] Moreover, a serological study showed that types 1 and 17 helper T-cell cytokines, which are usually associated with cell-mediated immunity and commonly linked to the pathogenesis of autoimmune/inflammatory diseases, could be an early host response signature in severe infections.[21] However, the role of the host immune response in human lung tissue, regarding the severe respiratory tract pathogenesis of 2009 pH1N1 infection, has remained unexplored.

In the present study, we investigated viral load, immune response, and the induction of apoptosis in the lung tissues from 50 fatal 2009 pH1N1 cases. The results suggested that the pathogenesis of the viral infection is associated with both viral replication and production of proinflammatory mediators. In addition, FasL and caspase-3 are involved in the apoptotic process frequently observed in lung and airway tissues of 2009 pH1N1 fatal cases. The disequilibrium between FAS and FasL levels in the lung could contribute to delayed clearance of virus and subsequent pathological damage.

Materials and Methods

Patient Lung Tissue Specimens

This study includes autopsy specimens collected from 50 confirmed 2009 pH1N1 case-patients, submitted to the Infectious Diseases Pathology Branch, Centers for Disease Control and Prevention (Atlanta, GA), for evaluation from May 2009 to February 2010. A confirmed case of 2009 pH1N1 was defined as a patient with influenza-like illness, and 2009 pH1N1 virus infection was confirmed by real-time RT-PCR or viral culture by either premortem or postmortem respiratory tract specimen testing. Demographic data, laboratory test results for influenza virus and bacteria, and other relevant clinical information were collected from medical records and preliminary autopsy reports, when available.

Unfixed fresh or frozen tissues, in addition to formalin-fixed tissues, were available for 48 of the cases in this study.

Study Design

Of 50 cases, 42 were evaluated by the viral load assay, because these cases had fresh tissues and information available on illness duration. A Bio-Plex cytokine assay (Bio-Rad, Hercules, CA) was performed on 27 (21 of the 42 cases used in the viral load assay and 6 cases with limited lung tissues only available for the Bio-Plex assay) of 50 cases based on the availability of fresh lung tissues, excluding those with a long duration of illness (>15 days), pregnancy, and a history of immunosuppression. The quantitative RT-PCR assays for determination of mRNA levels of cytokines and apoptosis-associated genes were performed on 32 cases that had lung tissues available for evaluation and were 2009 pH1N1 virus PCR positive in lung tissues. An immunostaining assay for localization of cytokines was performed on formalin-fixed, paraffin-embedded (FFPE) tissues from 13 cases with high cytokine levels, as determined by the Bio-Plex assay, and two cases that demonstrated extensively positive immunostaining for influenza nucleoprotein on lung section and only had formalin-fixed tissues available for evaluation.

Control Lung Tissue Specimens

The control group consisted of 12 normal lung tissue specimens (normal group). The lung tissues of the normal group were obtained from biopsy tissues archived by the Human Tissue Procurement Service of Pathology and Laboratory Medicine at Emory University School of Medicine (Atlanta, GA). The tissues were identified as normal lung tissue by routine H&E staining. No pathological changes of ill condition, including inflammation or tumor, were observed in the tissues.

Tissue Processing and Assays for Viral and Bacterial Agents

All fresh-frozen tissues from 2009 pH1N1 fatal cases were evaluated by the Centers for Disease Control and Prevention real-time RT-PCR protocol to determine whether the tissues were positive for 2009 pH1N1. The tissues were then fixed in 10% buffered formalin, processed for paraffin embedding, and stained by H&E for histopathological examination. For FFPE lung tissues, special stains, including Lillie-Twort Gram stain and Warthin-Starry silver impregnation stain, were used to determine the presence of a bacterial infection. As described in our previously published study,[22] IHC was performed for the detection of influenza A virus, group A streptococcus (*Streptococcus pyogenes*), *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*. As described previously,[22] the broad-range eubacterial PCR assay targeting the 16S rRNA gene and conventional single-stage or nested PCR assays for *S. pneumoniae*, *S. pyogenes*, and *H. influenzae* were performed

using DNA extracted from FFPE lung tissue specimens from the case-patients. Amplified PCR products were sequenced on an automated CEQ 8000 DNA Sequencer (Beckman Coulter, Fullerton, CA) for the confirmation of bacterial agents.

Bio-Plex Cytokine Assay

Cytokine and chemokine protein levels in frozen lung tissues were assessed by the 27-plex prohuman cytokine bead-based cytokine assay (Bio-Rad). Cytokines tested were as follows: IL-1β, IL-1 receptor antagonist protein (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon-γ, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-α (MIP-1α), MIP-1β, human platelet-derived growth factor BB, chemokine ligand-5/regulated on activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, vascular endothelial growth factor, and interferon-inducible protein-10 (IP-10). Based on the clinical and laboratory findings, 27 of 50 cases were evaluated by this assay (pH1N1 group), and were divided into two groups: pH1N1 nonbacterial co-infection group ($n = 20$) and pH1N1 with bacterial co-infection group ($n = 7$). Furthermore, the 12 normal lung tissues previously mentioned were used in the assay (normal group). Frozen lung tissues were homogenized in cell lysis buffer (Bio-Rad) containing a protease inhibitor mixture (Mintabs; Roche Applied Science, Indianapolis, IN), using a tissue weight (mg) to lysis buffer volume (µL) ratio of 1:4. The homogenates were lysed by alternate freeze ($-80°C$)/thaw cycles and sonicated for 10 minutes on ice, followed by centrifugation at $500 \times g$ for 15 minutes at $4°C$. For each sample, 50 µL of supernatant was used to measure cytokine levels using the Bio-Plex Pro Human Cytokine 27-plex Assay kit (Bio-Rad), according to the manufacturer's instructions using a Bio-Plex 200 array reader (Bio-Rad). Each sample was run in duplicate. For data acquisition, Bio-Plex Manager Software, version 4, was used (Bio-Rad).

RNA Extraction

RNA was extracted from fresh-frozen lung tissues using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) per kit protocol. Briefly, approximately 100 µg of lung tissue from different lobes was lysed in buffer RLT (Qiagen). RNA was obtained in a 50-µL volume after genomic DNA was removed by use of a genomic DNA spin column and stored at $-80°C$ before use.

Standard RNA Synthesis

In vitro transcribed RNA to the Matrix gene of pH1N1, human cytokines (including IL-1RA, IL-6, IL-8, MIP-1β,

MCP-1, IP-10, and TNF-α), and apoptosis-associated genes [Fas, FasL, and TNF-related apoptosis-inducing ligand (TRAIL)] was used to determine the detection limit of the assay and as an internal positive control. A recombinant plasmid containing the Matrix gene was linearized with restriction enzyme EcoRI and purified using Qiagen's DNA cleanup kit. DNA concentration was measured as OD units at 260 nm. Cytokines and apoptosis-associated gene segments were amplified by specific primers, with *T7* and *SP6* promoter sequences at the terminal ends. Linearized plasmid DNA or purified PCR product from cytokine (1 µg) and apoptosis-associated gene segments were transcribed using the Riboprobe *in Vitro* Transcription System kit (Promega, Madison, WI) by the *T7/SP6* promoter, according to the manufacturer's instructions. The transcribed RNA was purified and quantified by copy number. For normalization using the human housekeeping gene, β2-microglobulin (*B2M*), purified total RNA extracted from A549 cells was used.

Real-Time Quantitative RT-PCR

To quantify the influenza viral load and cytokine mRNA and apoptosis-associated mRNA expression in lung tissues, a real-time quantitative RT-PCR (RT-qPCR) was performed using the QPCR detection system (Agilent Technologies Inc., Santa Clara, CA) using a fluorescently labeled TaqMan probe to enable continuous monitoring of amplicon formation. The primers and probe for influenza A (Matrix gene) were previously described.[22] The cytokines (IL-6, TNF-α, IL-1RA, IL-8, MCP-1, and MIP-1β), apoptosis-associated (Fas, FasL, and TRAIL), and *B2M* primers and probes used in this study were as previously published.[23−28] The assays were performed in a total volume of 25 µL using the QuantiTect Probe PCR Kit (Qiagen), 5 µL total RNA, 40 µmol/L each primer, and 20 µmol/L each probe. The thermal cycling conditions used for the assays were as follows: reverse transcription at $50°C$ for 30 minutes, 1 cycle at $94°C$ for 15 minutes, then 45 cycles at $94°C$ for 15 seconds, $55°C$ for 30 seconds, and $72°C$ for 30 seconds each. A standard curve was generated using serial dilution of an *in vitro* transcribed standard RNA (from approximately 10 to 10^7 copies) or A549 cell RNA (from approximately 0.1 pg to 100 ng).

Apoptosis Detection

TUNEL staining was used for *in situ* detection of apoptosis in FFPE lung tissues from fatal cases. Detection was performed on deparaffinized FFPE sections (4 µm thick) with the Cell Death TUNEL POD Detection System (Roche), according to the manufacturer's instructions. An internal positive control consisted of FFPE sections taken from human colon tissue (Biocare Medical, Concord, CA).

Immunohistochemistry Data

To assess cytokine distribution in respiratory tract tissues, IHC was performed on deparaffinized FFPE sections (4 µm

Table 1 Characteristics of Fatal Cases of 2009 pH1N1 Infection

Characteristics	Value
All patients	50
Median age (mean, range)	38 (37.33, 4 months-72 years)
Female	28 (56%)
Bacterial co-infection	14 (28%)
S. pneumoniae	8
S. aureus	4
Group A streptococcus	2
Median duration (range)	8 (2–19 days)
Underlying medical conditions	34 (68%)
Asthma	7
Obesity	4
Down syndrome	2
Pregnancy	1
With other medical conditions	6
Immunosuppression*	2
$\geq$2 Conditions previously listed	12

Data are given as number of patients unless otherwise indicated.

*Including solid organ, stem cell, and bone marrow transplantation, HIV infection/AIDS, and drug dependence.

thick) using monoclonal/polyclonal antibodies against IL-6 (AF-206-NA; R&D Systems, Minneapolis, MN), IP-10 (sc-101500; Santa Cruz Biotechnology, Dallas, TX), IL-8 (PA1-36108; Thermo Scientific, Waltham, MA), and cleaved caspase-3 (CP229A; Biocare Medical) by using a polymer-based colorimetric indirect immunoalkaline phosphatase method. Positive controls consisted of human colon tissue (for cleaved caspase-3) and lymphocytes (HUT-78 and THP-1) treated by phytohemagglutinin and lipopolysaccharide. Negative controls consisted of sequential case patient tissue sections incubated with normal mouse or rabbit serum pertinent to the primary antibody. The antibody/polymer conjugate was visualized by applying fast red chromogen (LabVision, Waltham, MA) dissolved in naphthol phosphate substrate buffer to tissue sections.

Specificity of Cytokine/Chemokine Immunostaining

Blocking experiments that were described in a previous report[29] were performed to confirm the specificity of IL-6, IP-10, and IL-8. In brief, the antibodies previously mentioned were incubated with 500 ng/mL recombinant human IL-6, IP-10, and IL-8 for 1 hour at 37°C in diluent buffer containing Tris-buffered saline/10% normal mouse serum/0.1%Triton X-100. The untreated and preabsorbed antibodies were used as primary antibodies and incubated on normal lung tissues (negative control), cell controls (positive control), and 2009 pH1N1-infected lung tissues, as previously mentioned.

Statistical Analysis

The quantitative cytokine/chemokine profiles, apoptosis-associated gene mRNA levels, and viral load were compared

by U-test or Kruskal-Wallis test. The correlation between viral load and quantitative proinflammatory factors was analyzed by Pearson's correlation test. Differences were considered significant at $P < 0.05$ with a two-tailed test. All analysis was performed using Instat Vision version 5.0 (GraphPad Prism, La Jolla, CA).

## Results

Demographic Characteristics of Fatal Cases

The demographic data of the 50 cases are summarized in Table 1. The median age of the patients was 38 years (interquartile range, 4 months to 72 years), with a mean age of 37.33 years. Forty-three patients (86%) were in the age range of 15 to 59 years. Twenty-eight (56%) of the patients were female. In addition, 34 (68%) of the patients had a documented history of underlying medical conditions, including asthma, obesity, pregnancy, immunosuppression, or other complications.

### Viral Load and Duration of Illness

As shown in Figure 1, the viral load correlated with duration of illness ($P = 0.0247$). A relatively high viral load was present at the early stage of the illness, peaked on day 7 of the illness, and could be detected as late as day 17 after illness onset.

Cytokine/Chemokine Profiles

Compared with the normal control group, 7 of 27 evaluated cytokines/chemokines assayed were more highly expressed in lung tissues of 2009 pH1N1 fatal cases, including IL-6, TNF-α, IL-1RA, IL-8, MCP-1, MIP-1β, and IP-10 (P range, <0.0001 to 0.0030) (Figure 2). Furthermore, when stratified by individuals with the presence of a bacterial co-infection, IL-6 and TNF-α showed marked increases in the pH1N1 with bacterial co-infection group
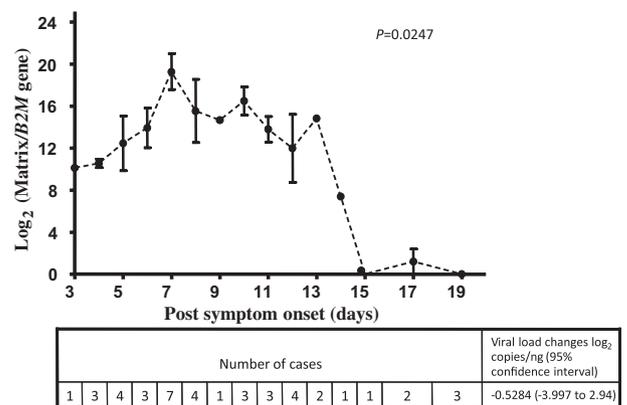
Figure 1 Viral load in fresh-frozen lung tissues, as measured by RT-qPCR. The diagram displays quantification of the matrix gene ($\log_2$ of the matrix gene/B2M housekeeping gene) in lung tissues of fatal cases over different durations. The Kruskal-Wallis test was performed to assess variance at different durations.
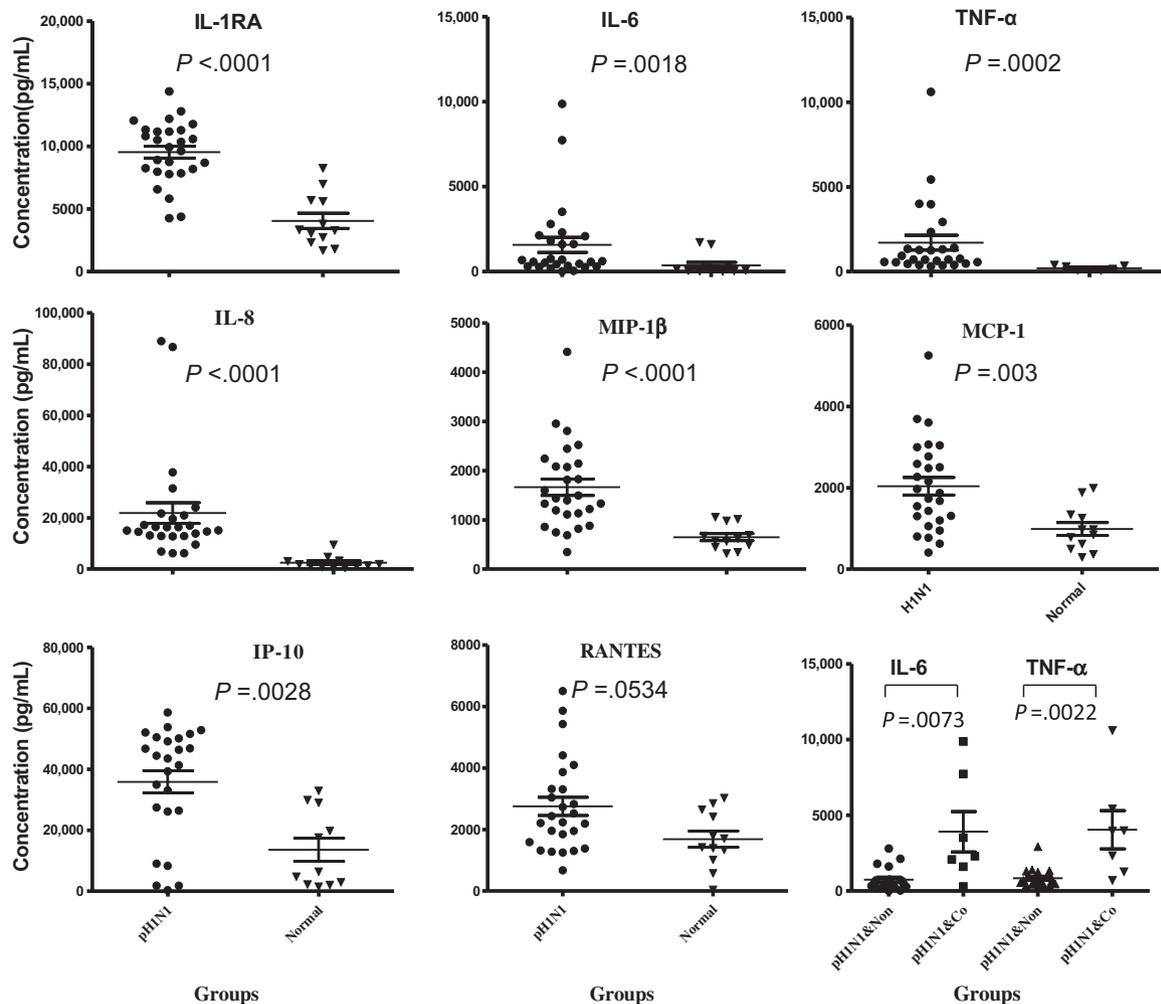
Figure 2   Profiles of cytokines and chemokines in lung tissues. The graphs depict the levels of IL-1RA, IL-6, TNF-α, IL-8, MIP-1β, MCP-1, IP-10, or RANTES protein expression in pH1N1, normal, pH1N1 with bacterial co-infection, or pH1N1 with nonbacterial co-infection groups. After fresh-frozen lung tissues were homogenized in lysis buffer, the supernatants were tested using a 27-cytokine magnetic bead-based array. Error bars represent means ± SEM. A *U*-test was performed to assess statistical significance.

($P = 0.0073$ and $P = 0.0022$, respectively) compared with the pH1N1 nonbacterial co-infection group. RANTES was not significantly higher in the pH1N1 group ($P = 0.0534$).

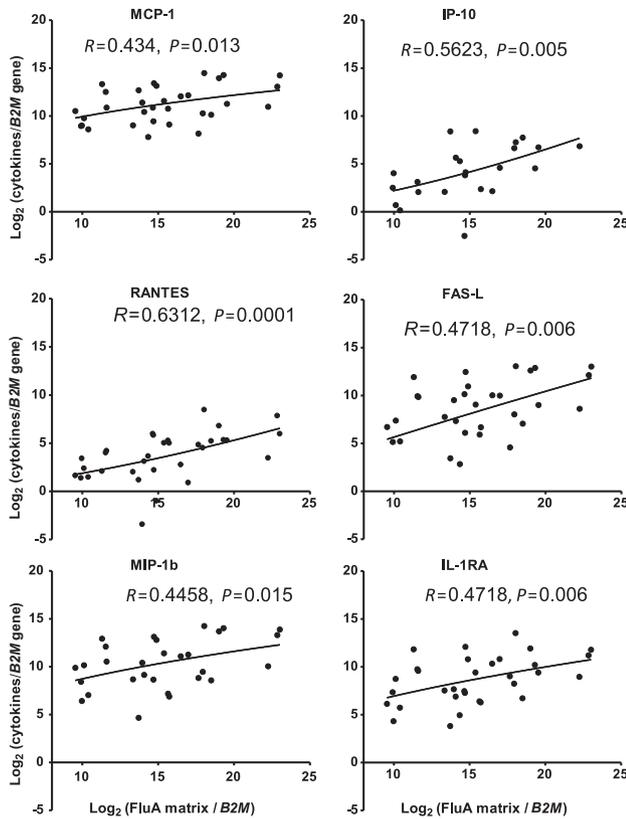### Correlation between Viral Load and Cytokine mRNA Levels

To analyze the correlation between viral load and host response, fresh-frozen lung tissues from 32 pH1N1 influenza real-time PCR-positive cases were tested using RT-qPCR assays designed to quantify expression levels of immune mediator mRNAs, including IL-6, TNF-α, IL-1RA, IL-8, MCP-1, MIP-1β, IP-10, and RANTES. Pearson's correlation test suggested that, as shown in Figure 3, viral load was positively correlated with IL-1RA, IL-6, MCP-1, MIP-1β, IP-10, and RANTES (with correlation coefficients ranging from 0.42 to 0.54, $P = 0.0018$ to 0.0216) (Supplemental Table S1). IL-8, IL-6, and TNF-α mRNA levels were not significantly correlated with viral load ($P = 0.5426$, 0.3889, and 0.7456, respectively).

Apoptosis-Associated Gene Assay

As shown in Figure 4, FasL mRNA level in lung tissue of 2009 pH1N1 fatal cases was remarkably higher than in normal lung controls ($P = 0.0048$). In addition, the FasL mRNA level was positively correlated with the viral load in lung tissue ($P = 0.0053$) (Figure 3 and Supplemental Table S2). However, low Fas mRNA levels were detected in the lung tissue from only 6 of 32 cases, and compared with the normal group, H1N1 infection resulted in significantly lower Fas mRNA levels ($P = 0.0065$). Furthermore, TNF-related apoptosis-inducing ligand (TRAIL) mRNA levels were not significantly different between the two groups ($P = 0.3312$).

Distribution of the Immune Mediators

As mentioned in our previous report,[22] diffuse alveolar damage (Figure 5A), infiltration of inflammatory cells in glands (Figure 5B), and bronchopneumonia (Figure 5C) were frequent histopathological findings in 2009 pH1N1 fatal

Figure 3 Correlation between viral load and immune mediator mRNA levels. Relative quantification of gene expression (log$_2$ scale) was normalized to that of the *B2M* housekeeping gene for the viral matrix gene and immune mediator mRNAs (MCP-1, IP-10, RANTES, FasL, MIP-1β, and IL-1RA).

cases. Viral nucleoprotein was localized in the nuclei and cytoplasm of pneumocytes, lining alveoli, or glandular epithelial cells (Figure 5, D and E). To establish a correlation with viral infection or pathological change, the distribution of immune mediators in lung was demonstrated by IHC staining with antibodies against IL-6, IL-8, and IP-10. These antibodies were confirmed to work on FFPE tissues stained with the IHC assay by testing relevant cell controls (Supplemental

Figure S1). The specificity of these antibodies was demonstrated by a blocking assay on positive cases using corresponding recombinant protein (Figure 5, I, L, and O) and normal lung tissues as a negative control (Figure 5F). The immune mediator-producing cells were detected in lung and submucosal tissues with varying degrees of distribution. Immunostaining for individual cytokines and viral antigen was performed using consecutive sections. IL-6, IL-8, IP-10, and viral antigen were localized in similar areas within pneumocytes and submucosal glands of patients with bacterial co-infection (Figure 5, G–N). Similar results were observed in non–co-infected patients. The photomicrographs shown in Figure 5 were from patients with co-infections (a 47-year-old woman after a 7-day clinical course of 2009 pH1N1 infection and group A streptococcus co-infection and a 7-year-old girl after a 5-day duration of illness and *S. pneumoniae* co-infection).

## Apoptosis and Apoptosis-Associated Caspase-3 Detection

The IHC assay demonstrated prominent expression of cleaved caspase-3 in submucosal glands (Figure 6A), pneumocytes, and epithelial cells in areas of bronchopneumonia (Figure 6, C and E), alveoli (Figure 6G), and lymphoid tissue (Figure 6I). TUNEL staining suggested that apoptotic cells were extensively present in lung and airway. The apoptotic bodies were corresponded with cleaved capase-3 immunostaining in submucosal glands (Figure 6B), pneumocytes, and epithelia in areas of bronchopneumonia (Figure 6, D and F), alveolar pneumocytes (Figure 6H), and lymphoid tissues (Figure 6J) in consecutive tissue sections.

Discussion

Fatal cases of 2009 pH1N1 virus infection present with different characteristics from seasonal influenza virus infections, including age distribution patterns with respect to morbidity[11] and lung pathological characteristics.[3,30] In seasonal influenza
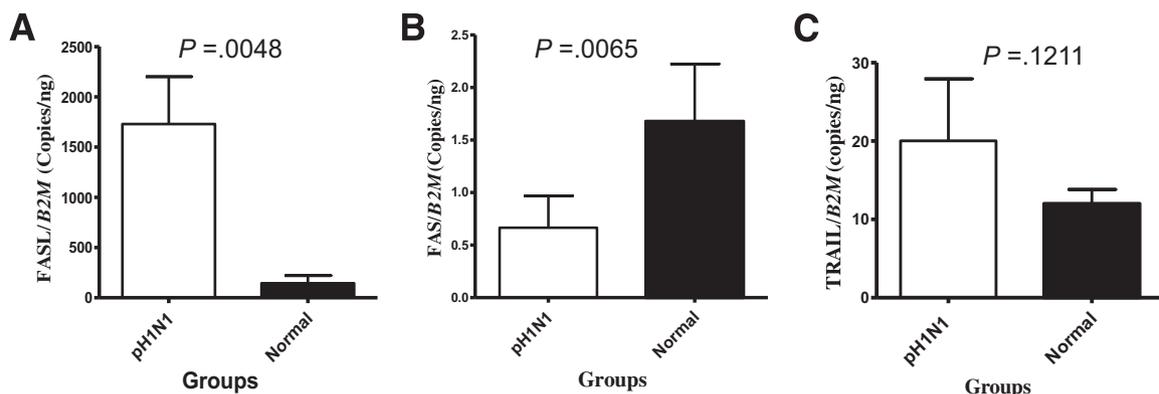
Figure 4 Apoptosis-associated gene mRNA levels, as determined by RT-qPCR. The column diagrams depict the relative quantification of FasL (**A**), FAS (**B**), and TRAIL (**C**) mRNA in lung tissues. Error bars represent means ± SEM. The *U*-test was performed to assess the statistical significance between 2009 pH1N1 cases and the normal group.
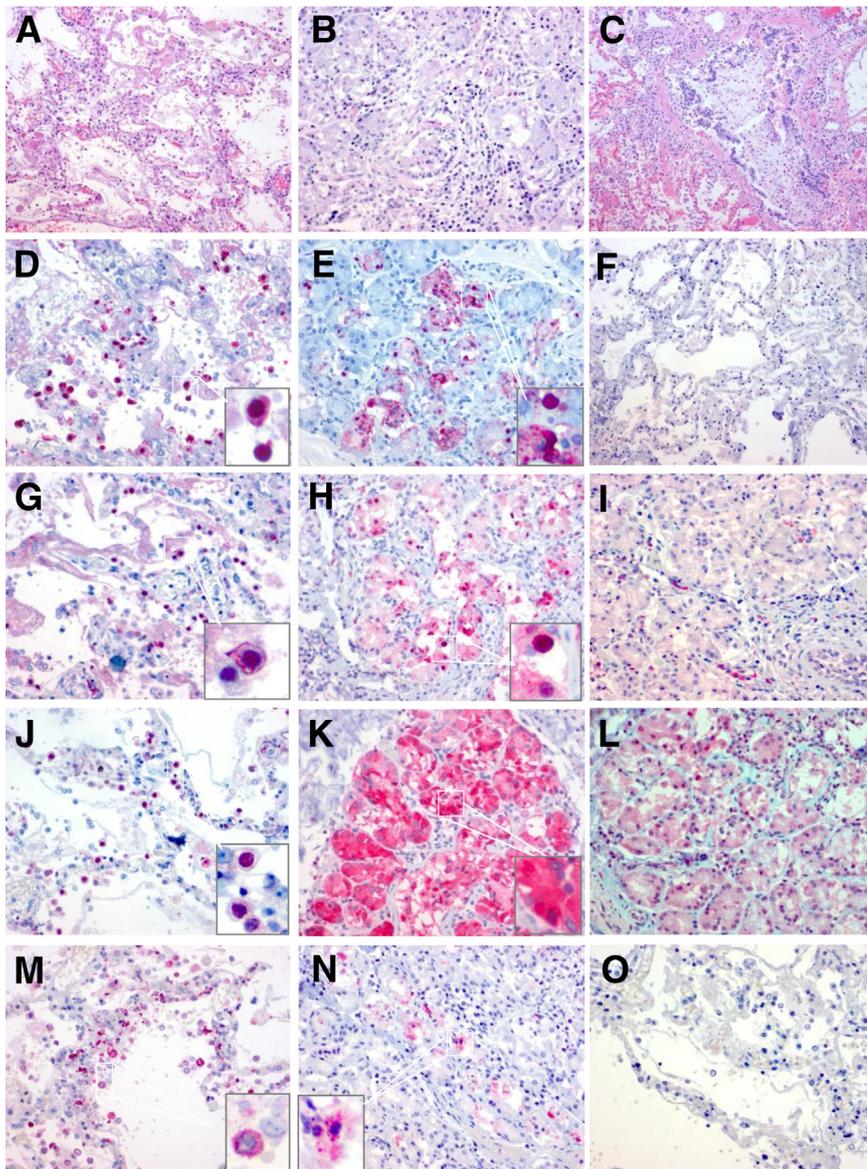
Figure 5 Histopathological characteristics and immunostaining of viral antigens and cytokines in lung and airway. Diffuse alveolar damage (**A**), infiltration of inflammatory cells in glands (**B**), and bronchopneumonia (**C**) using H&E staining. Viral nucleoprotein immunohistochemical staining (red) in nuclei and cytoplasm of pneumocytes lining alveoli (**D**) and submucosal gland cells (**E**). Negative IHC staining for IL-6 in normal lung tissue (**F**), IL-6 immunostaining (red) in nuclei and cytoplasm of pneumocytes (**G**) and submucosal gland cells (**H**), and after recombinant IL-6 absorption (**I**). IP-10 immunostaining (red) was present in the nuclei and cytoplasm of pneumocytes (**J**) and submucosal gland cells (**K**), and after recombinant IP-10 absorption (**L**). IL-8 immunostaining (red) present in the cytoplasm of pneumocytes (**M**) and submucosal gland cells (**N**), and after recombinant IL-8 absorption (**O**). Original magnifications: ×10 (**A—C**, **F**, **I**, **L**, and **O**); ×20 (**D**, **E**, **G**, **H**, **J**, **K**, **M**, and **N**); ×100 (**insets D**, **E**, **G**, **H**, **J**, **K**, **M**, and **N**).

virus infection, the replication of virus in the respiratory tract directly injures the nasal and tracheobronchial epithelium, and has been associated with virus-induced cellular apoptosis. The resulting involvement of respiratory tract epithelial cells is one of the major reasons for several manifestations that accompany infection, such as cough, depressed tracheobronchial clearance, and altered pulmonary function.[31] Infection also elicits a cascade of host immune defenses leading to local inflammation and an influx of polymorphonuclear cells, lymphocytes, and macrophages into the respiratory tract mucosa and lung.[32] Although this response leads to resolution of the infection and protection against reinfection, it is likely that it also contributes to the development of local and systemic symptoms. In addition, it has traditionally been well documented that danger to the host is generated when infection by an influenza virus predisposes to secondary infections with bacterial pathogens.[33] This study was designed to evaluate the role of viral load and distribution, cytokine response, and apoptosis in the pathogenesis of 2009 pH1N1 virus infection.

The clinical significance of prolonged viral shedding and the relatively late peak of viral kinetics are not completely understood. Studies suggest that initial nasopharyngeal and endotracheal viral loads peaked 2 to 3 days after symptom onset, irrespective of disease severity,[34] and that prolonged viral shedding was present.[35] However, pathological studies of 2009 pH1N1 virus infection showed that the virus predominantly infected the lung parenchyma.[12,22] To our knowledge, the viral load and viral shedding in lung tissues have not yet been sufficiently assessed. In the present study, our results show that viral load in lung tissues can be detected as late as day 17 after the onset of illness and peaks on day 7 after onset. This finding could be related to viral infection of the lung parenchyma, because patients with pneumonia had higher viral loads than those with bronchitis or upper respiratory tract infection.[36] This could also be explained by a less
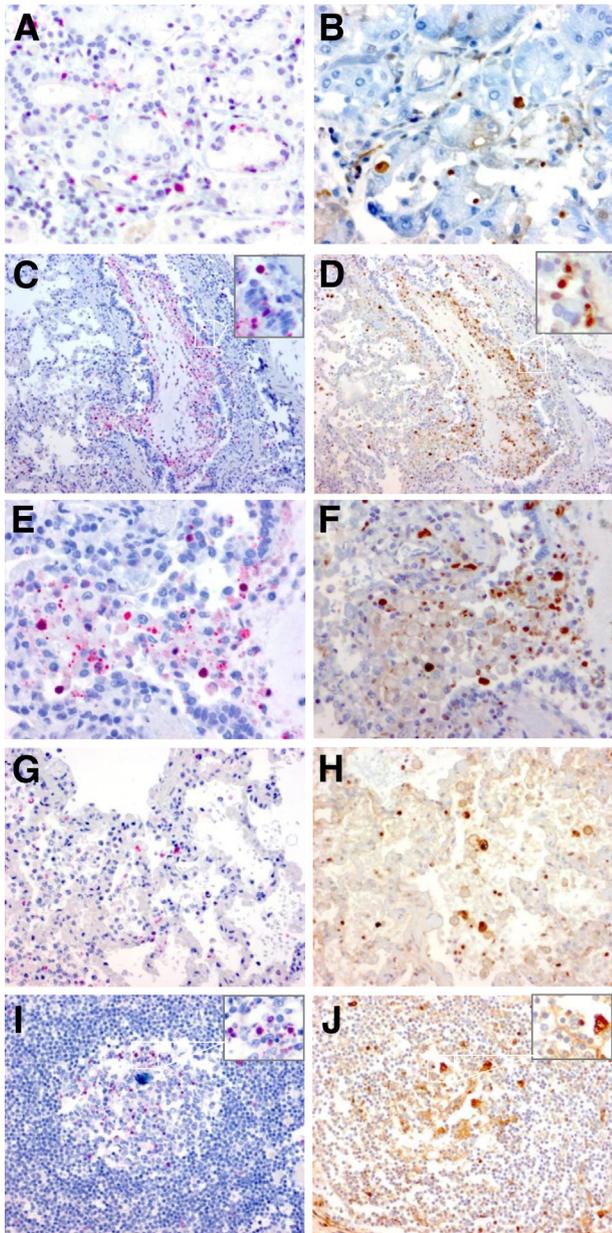
Figure 6 Cleaved caspase-3 immunostaining and TUNEL assay on consecutive sections of lung and airway. Caspase-3 immunostaining (red) demonstrates cleaved capase-3 staining in nuclei and cytoplasm of submucosal glands (**A**), epithelium and pneumocytes (**C** and **E**), alveoli (**G**), or lymphoid tissue (**I**). By TUNEL assay, apoptotic bodies (brown) seen in submucosal glands (**B**), epithelium and pneumocytes (**D** and **F**), alveoli (**H**), or lymphoid tissue (**J**). Original magnifications: ×10 (**C** and **D**); ×20 (**A**, **B**, **G**, **H**, **I**, and **J**); ×40 (**E** and **F**); ×100 (**insets C**, **D**, **I**, and **J**).

effective innate and adaptive immune response, combined with a delay in starting antiviral therapy due to a late presentation of symptoms, because antiviral therapy can shorten the duration of viral shedding if prescribed during the first 3 days of illness.[37]

Our study demonstrated a remarkable elevation of IL-1RA, IL-6, IL-8, TNF-α, MCP-1, MIP-1β, and IP-10 in 2009 pH1N1 fatal cases, which correlated with the peak

viral load in lung tissues. Interestingly, IL-8 mRNA levels were not positively correlated with viral load, although IL-8 is present in significant levels in lung tissues from 2009 pH1N1 fatal cases. The result could be explained by the nature of IL-8 and its function in influenza. IL-8 is a potent promoter of polymorphonuclear cell movement from blood into tissues, where these cells release mediators that can be harmful and may persist for relatively long periods after stimulation.[38] However, IL-8 is not a potential mediator of the measured symptoms and signs of influenza A infection.[39] In addition, unlike previous seasonal influenza studies,[40] RANTES levels in 2009 pH1N1 fatal cases were not significantly different from the normal lung group. However, viral load was positively correlated with RANTES mRNA level in lung tissues. Thus, it can be reasonably assumed that the virus may effectively induce RANTES expression in lung because mRNA can be detected more extensively than the corresponding cytokine, playing a physiological role during the viral infection. RANTES, a chemokine, plays an active role in recruiting leukocytes into inflammatory sites and induces the proliferation and activation of certain natural killer cells to form chemokine-activated killer cells.[41] Previous studies have shown that RANTES is associated with an increase in the efficiency of virus-cell fusion.[42]

IL-6 and TNF-α could be specifically associated with bacterial co-infection. Previous studies showed that elevated IL-6 and TNF-α were present in severe influenza virus infections,[34,43,44] and high levels correlated with systemic disease symptoms associated with influenza virus infections.[45] IL-6 may have a role in initiating the measured symptoms and signs of infection. With respect to both cytokines, our study is in agreement with previous studies that showed these cytokines were elevated in lung tissues. However, when stratified by the presence of a bacterial co-infection, our evidence suggests that IL-6 and TNF-α are more associated with pH1N1 cases having a bacterial co-infection than those with pH1N1 virus infection alone.

Viral pathogenesis in severe infections may be associated with both the viral replication and potentially an exacerbated host immune response. A previously published study suggested that parenchymal lung involvement of the 2009 pH1N1 virus is similar to the histopathological features and viral localization seen in fatal human case-patients of highly pathogenic avian influenza (H5N1).[22] Studies have suggested that a cytokine storm occurs in highly pathogenic avian influenza (H5N1) cases,[17,18] and the high virulence of H5N1 virus is associated with increased host responses.[46] However, the 2009 pH1N1 virus was thought to be a less virulent virus than H5N1, because it induces mild illness in 8% to 32% of infected persons.[47] In this study, our evidence suggests that a virus-induced immune response is present in the lungs of fatal cases, as demonstrated by increased levels of cytokines/chemokines, including IL-1RA, IL-6, TNF-α, IL-8, MIP-1β, MCP-1, and IP-10. IL-1RA is generally thought to be a potent anti-inflammatory cytokine. However, the balance of IL-1RA and IL-1 levels in local tissues influences the physiological or

pathophysiological effects.[48] Some studies have suggested that IL-1RA has varying inflammatory effects, and IL-1RA levels are positively associated with the severity of certain infectious diseases.[49−51] In addition to IL-6, IL-8, and TNF-α previously mentioned, MIP-1β, MCP-1, and IP-10 can play important roles in the innate immune response that is crucial for the initiation and subsequent direction of adaptive immune responses, as well as in promoting localized inflammation and producing cytokines that recruit additional leukocytes to the site of infection. However, unrestrained or excessive stimulation of the innate immune response can be harmful. Furthermore, the levels of immune mediators in the lung are positively correlated with viral load. Our evidence suggests that the virus can replicate efficiently and achieve a high viral load in the lung at an early stage of infection. Immunostaining for individual cytokines and viral antigens, performed on consecutive sections, showed that IL-6, IL-8, IP-10, and viral antigen were localized in similar areas within lung and submucosal glands. Monokines (IL-6 and IL-8) were detected in both small cells within alveolar walls and larger cells in the alveolar air spaces, whereas lymphokine IP-10 was seen mainly in small cells located within the alveolar walls. The presentation of these cytokines/chemokines is consistent with the documented results that CD8^{+} and CD68^{+} immune cells are extensively present in lung tissues,[22,52] and may activate the complement system in lung tissues of 2009 pH1N1 fatal cases,[52] resulting in an exacerbated host immune response. Taken together, we conclude that the virus can replicate efficiently in lung, and viral pathogenesis is related to both viral replication and production of proinflammatory mediators.

Apoptosis is obvious in the lungs of 2009 pH1N1 fatal cases, and FasL and caspase-3 are involved in the apoptotic pathway. Influenza-induced apoptosis has been found in a variety of cell types both *in vitro* and *in vivo*.[53−55] However, the precise mechanism of virus-induced apoptosis is unclear. Currently, there are two major pathways of apoptosis induction. The intrinsic pathway is regulated by the activation or interaction of members of the Bcl-2 family. This was postulated to be a host defense mechanism, by stopping the replication and spread of virus. The extrinsic pathway of apoptosis is triggered by certain TNF family members when they engage their respective cognate receptors on the surface of the target cells. In addition to TNF itself and TRAIL, Fas/FasL belongs to a subfamily of ligands that are responsible for extrinsic induction of cell death. Our results suggested that apoptosis is present in lung tissues of 2009 pH1N1 fatal cases, and mRNA levels of the *FasL* gene, which are positively correlated with viral load, are remarkably elevated in lung tissues; however, TRAIL mRNA levels were not significantly increased. In addition, the expression of the *FAS* gene was inhibited in lung tissues. Animal model studies have suggested that FasL is involved in viral clearance and in the development of immunopathological characteristics after respiratory tract virus infection,[56−59] and mice with a deficiency in functional FasL exhibit reduced morbidity after influenza virus infection compared with wild-type mice.[58]

However, our results suggest that in fatal human pH1N1 cases, elevated FasL levels trigger the induction of apoptosis in lung tissues, resulting in severe pathological characteristics, and the disequilibrium between Fas and FasL could delay viral clearance. In addition, the common event in most pathways of influenza virus-induced apoptosis is the activation of a set of cysteine proteases (caspases).[60] In this study, colocalization of cleaved caspase-3 with apoptotic bodies can be seen in both lung and airway of 2009 pH1N1 fatal cases, although the expression of other subtypes of caspases was not studied. The results indicate that caspase-3 is involved in the pathway of 2009 pH1N1-induced apoptosis in fatal cases.

In conclusion, we investigated viral load, the immune response, and apoptosis association in autopsied lung tissues from fatal 2009 pH1N1 cases. Our results indicate that viral load with prolonged viral shedding peaked on day 7 after illness onset in lung tissues, and 2009 pH1N1 can induce an exacerbated local immune response and apoptosis. The pathogenesis of the virus infection is associated with both viral replication and production of proinflammatory mediators in respiratory tract tissues. In addition, FasL and caspase-3 are involved in the pathway of 2009 pH1N1 virus-induced apoptosis in lung tissues, and the disequilibrium between Fas and FasL levels in the lungs could contribute to delayed clearance of virus and pathological damage. As such, this has considerable implications in the development of clinical treatment strategies.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.06.023*.

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