Hypercytokinemia and Hyperactivation of Phospho-p38 Mitogen-Activated Protein Kinase in Severe Human Influenza A Virus Infection

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Background. We postulate that hypercytokinemia plays a role in immunopathogenesis of severe human influenza.

Methods. We prospectively studied 39 consecutive patients who were hospitalized with severe influenza A virus infection. On laboratory confirmation of the diagnosis, paired acute-phase (obtained at hospital admission) and convalescent-phase (obtained 10 days after hospital admission) plasma samples were collected for assay of 11 cytokines and chemokines (interleukin [IL] 1β; IL-6; IL-10; IL-12p70; tumor necrosis factor α; IL-8; monokine induced by interferon [IFN]-γ; IFN-inducible protein 10; monocyte chemoattractant protein 1; regulated upon activation, normal T cell–expressed and secreted; and IFN-γ) using cytometric bead-array analysis and enzyme-linked immunosorbent assay. Simultaneously, virus concentration in the acute-phase nasopharyngeal aspirate was determined using real-time quantitative reverse-transcriptase polymerase chain reaction. Intracellular signaling molecules regulating lymphocyte activation, phospho-p38 mitogen-activated protein kinase and phospho-extracellular signal-regulated protein kinase in CD4+ and CD8+ T lymphocytes were studied in the acute-phase samples using flow cytometric analysis and were compared with results for samples from healthy control subjects.

Results. Statistically significant increases in plasma IL-6 (3.7-fold increase), IL-8 (2.6-fold increase), IFN-induced protein 10 (4.9-fold increase), and monokine induced by IFN-γ (2.3-fold increase) concentrations were detected during acute illness (P < .01 for all, by Wilcoxon signed-rank test); the highest concentrations were observed on symptom days 3 and 4. Corresponding plasma cytokine and chemokine concentrations and nasopharyngeal viral loads showed statistically significant correlations (r = 0.41, 0.49, 0.54, and 0.46, respectively; P < .01). Phospho-p38 mitogen-activated protein kinase expression in CD4+ lymphocytes was increased, correlating with cytokine concentrations (e.g., for IFN-induced protein 10, r = 0.78; P < .01); phospho-extracellular signal-regulated protein kinase was suppressed. Advanced age and comorbidity were associated with aberrant IL-6, IL-8, and monokine induced by IFN-γ responses (P < .05, by Mann-Whitney U test). An elevated IL-6 concentration was independently associated with prolonged hospitalization (hospitalization for >5 days; P = .02), adjusted for age, comorbidity, and virus load.

Conclusions. Hypercytokinemia (of proinflammatory and T helper 1 cytokines) is detected in severe influenza, correlating with clinical illness and virus concentration. Hyperactivation of phospho-p38 mitogen-activated protein kinase (in T helper cells) is possibly involved. Early viral suppression may attenuate these potentially deleterious cytokine responses.

Influenza is estimated to be responsible for >226,000 hospital admissions annually in the United States [1]. In Hong Kong, the estimated figure is 13–58 hospital admissions per 10,000 elderly persons [2]. The risks for developing complications (e.g., respiratory or cardiovascular complications) and death due to influenza are highest among persons at the extremes of age and those with comorbidities [3, 4]. However, the pathogenesis of these severe influenza infections remains poorly understood [5, 6].

Studies of influenza A virus subtype H5N1 disease and the clinical features of the 1918 pandemic influenza (now known to be avian in origin) suggest that immunopathogenesis may play an important role in these
deadly infections [7, 8]. The syndrome of acute respiratory distress, multiple-organ dysfunction, lymphopenia, and hemophagocytosis is likely to be related to cytokine dysregulation ("cytokine storm") [8]. Grossly elevated serum concentrations of IL-6 and IFN-γ and the chemokines IFN-inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), and IL-8 have been detected in patients infected with the H5N1 strain [9-11]. Mechanistic studies indicate that the H5N1 strain potently induces gene transcription of proinflammatory cytokines and chemokines from macrophages and bronchial epithelial cells in vitro [12–14]. Human influenza viruses (influenza A subtypes H3N2 and H1N1) may similarly induce cytokine gene transcription in these cell types, although they appear to be less potent [6, 15, 16].

Few clinical studies on immunopathogenesis have been performed for human influenza infections. In mild, uncomplicated H1N1 infections (both experimental and naturally occurring) involving previously healthy individuals, plasma and nasal IL-6 concentrations have been found to be elevated and to correlate with viral titers, as well as with systemic and respiratory symptoms [17–19]. Significant releases of IL-8, TNF-α, IFN-α, IFN-γ, and IL-10 into nasal fluid or peripheral blood have also been detected [18–20]. However, certain chemokines (e.g., IP-10) and the cytokine and chemokine profiles in severe, complicated cases of influenza have not been studied systematically [6, 21–24].

Recent studies have shown that certain intracellular signaling mechanisms may be important in regulating the hyperproduction of cytokines and chemokines during acute influenza and other respiratory viral infections [6, 25–27]. One of these molecules, the phospho-p38 mitogen-activated protein kinase (MAPK), has been shown to contribute to hyperinduction of TNF-α in infections due to the H5N1 strain and to T cell–mediated immune responses to influenza virus infection [25, 26]. Differential activation of intracellular phospho-p38 MAPK in lymphocyte subsets for human influenza infection has not been studied in vivo.

In this study, we postulate that, in severe, complicated human influenza infections, dysregulation of extracellular cytokines can be detected in vivo, which may have virological and clinical correlations. Changes in plasma cytokine and chemokine concentrations during acute illness, the corresponding viral load, and possible intracellular signal transduction pathways are reported. Knowledge of the pathogenesis of severe influenza may help to improve clinical management.

**PATIENTS AND METHODS**

**Patients.** To investigate cytokine responses in the acute phase of severe influenza, we conducted a prospective observational study involving consecutive laboratory-confirmed adult (age, ≥18 years) patients with influenza who were admitted to the medical department of the Prince of Wales hospital (Hong Kong, People’s Republic of China) during the 2006 influenza season (1 February–31 July 2006) [28]. Hospitalized patients with influenza received a diagnosis and had their cases managed according to a standard protocol that included tests for common viral and bacterial respiratory pathogens [29]. In brief, all adult patients presenting with acute febrile respiratory illness requiring hospitalization were admitted to designated medical wards and placed on droplet precautions. Patients were admitted to the hospital if they developed potentially serious medical conditions or if the exacerbation of their chronic underlying illnesses or severe symptoms were considered to be unmanageable at home. Nasopharyngeal aspiration (NPA) was performed at admission to test for influenza A and B infections using immunofluorescence assay; the results were available within several hours.

Patients were recruited into the study if their NPA sample had immunofluorescence assay results positive for influenza A virus. To determine changes in cytokine and chemokine concentrations, 12-mL EDTA venous blood samples were taken for each patient (1) at admission, at the time of case recruitment, and within the day of initiation of antiviral treatment; and (2) at least 1 week after the symptoms of acute illness had subsided (the convalescent-phase samples). Simultaneous pretreatment influenza virus concentration in the NPA sample was also measured. Patients’ medical conditions were managed and patients were discharged from the hospital according to usual clinical practice, because the study did not have an intervention arm [29, 30]. Informed consent was obtained from all patients. Ethics approval for this study was obtained from the institutional review board of Prince of Wales Hospital.

**Virological investigations.** NPA samples were collected using a standard technique with appropriate infection-control measures [31]. The NPA sample was used for immunofluorescence staining, virus isolation, and influenza viral RNA quantitation; all samples were processed in a biosafety level II laboratory. A commercial immunofluorescence assay for influenza A and B viruses (Chemicon International) was used for the initial diagnosis of influenza. Influenza virus isolation was conducted using Madin-Darby canine kidney cells, and cell monolayers were examined daily for cytopathic effect. After 14 days of incubation, the growth of influenza virus was detected using hemadsorption and confirmed by immunofluorescence staining using influenza group–specific antibodies (Chemicon International), which identified the isolate as either influenza A virus or influenza B virus. Influenza A virus isolates were further differentiated into H1 and H3 subtypes by the National Influenza Reference Laboratory at the Centre for Health Protection in Hong Kong [28].

To estimate the amount of influenza A virus RNA in the
NPA samples, total RNA was extracted from the supernatants of the specimen using a QIAamp Viral RNA extraction kit (Qiagen) according to the manufacturer’s instructions (Appendix; online only). The resulting complementary DNA products were used immediately for real-time PCR. The primers used were 5′-AAG ACC AAT CCT GTG ACC TCT GA-3′ (forward primer) and 5′-CAA AGC TAC GTC GCC GCA GTC C-3′ (reverse primer), which amplified a 74-bp fragment in the M gene of influenza A virus [32]. These primers were designed to detect the influenza A virus RNA from all subtypes of human influenza viruses, including the most recently circulating viruses, H1N1 and H3N2. The influenza A virus–specific fluorescent probe, labeled with 6-carboxyfluorescein at the 5′ end and quencher dye 6-carboxytetramethyl-rhodamine at their 3′ end (6-carboxyfluorescein–5′-TTT GTG TTC ACG CTC ACC GT-3′–6-carboxytetramethyl-rhodamine), was designed to anneal to an internal sequence of the amplified region [32]. A 25-μL RT-PCR mixture consisted of 12.5 μL of 2X Taq Man Universal PCR Master Mix (Applied Biosystems), 0.2 μmol/L of each influenza A virus–specific primer, and 0.1 μmol/L of probe, plus 2 μL of DNA template. Amplification and detection were performed under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The real-time PCR reactions were carried out in a 96-well microtiter plate using ABI7900 (Applied Biosystems). Plasmids containing the known copy number of amplification targets were included in the real-time PCR assay to generate a standard curve for quantification of clinical samples.

**Measurement of plasma cytokines and chemokines.** EDTA blood samples were collected as described. They were immersed in ice and transported immediately to a biosafety level II laboratory for processing. Plasma was separated by centrifugation (2000 g for 10 min) at 4°C and stored in 300 μL aliquots at −70°C until analysis. Inflammatory cytokines IL-1β, IL-6, IL-10, IL-12p70, and TNF-α and chemokine IL-8, MIG, IP-10, monocye chemotactrant protein 1 (MCP-1), and regulated upon activation, normal T cell–expressed and secreted (RANTES) were measured simultaneously by bead-based multiplex flow cytometry using human inflammatory cytokine and chemokine bead array reagents, respectively (4-color FACSCalibur flow cytometer; BD Biosciences). In cytometric bead array measurement, different bead populations with distinct fluorescence intensities are coated with capturing antibodies specific for different cytokines or chemokines. After incubation with 50 μL of plasma, the beads are mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. Fluorescence flow cytometry of the beads provides simultaneous quantification of a panel of cytokines and chemokines. Plasma concentrations of T helper (Th) type 1 cytokine IFN-γ were quantified using an ELISA kit (R & D Systems). The assay sensitivities of IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, TNF-α, IL8, RANTES, MCP-1, IP-10, and MIG were 7.1, 2.5, 3.3, 3.7, 1.9, 7.2, 0.2, 1.0, 2.7, 2.8, and 2.5 pg/mL, respectively. Coefficients of variation were all <10%. Their respective reference ranges were derived from the measurement of samples from >100 healthy control subjects [33].

**Analysis of intracellular activated MAPKs.** To examine a possible signal transduction pathway in severe human influenza in vivo, acute-phase blood samples from the first 12 patients to be recruited (obtained in February and March 2006) were studied. Blood samples from 14 age- and sex-matched healthy subjects were used as controls. Differential activation of MAPKs in Th cells (CD4+ cells) and cytotoxic T cells plus suppressor T cells (CD8+ cells) from patients and control subjects were studied by flow cytometric analysis of intracellular phospho-p38 MAPK and phospho–extracellular signal-regulated protein kinase (ERK). In brief, PBMCs were prepared by centrifuging EDTA venous blood using a Ficoll–Paque density gradient (Amersham Pharmacia Biotech). The viability of PBMCs was >95%, as determined by trypan blue exclusion method. PBMCs were then fixed by BD Cytofix Buffer (BD Biosciences) at 37°C for 10 min. Cells were then permeabilized with BD PhosFlow Perm Buffer III for 30 min on ice and washed twice with BD Pharmingen Stain Buffer (BD Biosciences) and resuspended in BD Pharmingen Stain Buffer at 1 × 107 cells/mL. Fluorochrome-conjugated anti-human phospho-ERK, phospho-p38 MAPK, or mouse IgG isotypic antibody (BD Pharmingen) was added to each tube and incubated at room temperature for 30 min in the dark. Th cells and cytotoxic T cells plus suppressor T cells were then washed and resuspended for flow cytometric analysis using CD4+ and CD8+ cell gating, respectively (BD FACSCalibur flow cytometer; BD Pharmingen). Results were expressed as mean fluorescence intensity for intracellular phospho-ERK and phospho-p38 MAPK of 10,000 cells [27, 34].

**Statistical analysis.** Concentrations of cytokines and chemokines (expressed in picograms per milliliter) and influenza virus RNA concentrations (expressed in copies per milliliter) were log transformed for statistical analyses. Changes of cytokine and chemokine concentrations in the paired plasma samples were compared using the Wilcoxon signed-rank test. Spearman’s rank correlation coefficient was used to assess correlations between corresponding cytokine and chemokine concentrations and the viral RNA concentrations. The Mann-Whitney U test was used to test for univariate associations between cytokine and chemokine concentrations and clinical variables and to compare signaling molecule expression in patients with influenza with signaling molecule expression in healthy control subjects. To identify independent factors associated with more-severe illness (as indicated by hospitalization >5 days in duration; based on the results of our previous study [29]), variables with a P value <.1 in the univariate analysis were entered into logistic regression models together with baseline chrarac-
teristics. In all analyses, a P value of <.05 was considered to indicate statistical significance. All probabilities were 2-tailed. Statistical analysis was performed using SPSS software, version 13.0 (SPSS).

RESULTS

Patients with influenza. In total, samples from 39 patients were studied (table 1). All immunofluorescence assay results positive for influenza A virus were confirmed by virus isolation, and all virus isolates were subsequently identified as H1N1, the predominant circulating strain during the study period [28]. The mean age of the patients (±SD) was 57.1±21.1 years (median age, 57 years), and comorbidities were noted in 56.4% of the patients. Seventy-four percent of the patients developed acute illness (for all, by Wilcoxon signed-rank test). The increase in MCP-1 was also observed. In symptom days 3 and 4, and concentrations gradually normalized during convalescence (figure 1; online only). A small increase in the concentration of MCP-1 was also observed. In contrast, RANTES concentration was noted to increase during convalescence. There was no significant change in IFN-γ, IL-1β, IL-10, IL-12p70, and TNF-α concentrations detected.

Corresponding acute-phase plasma cytokine and chemokine concentrations and virus concentrations in NPA samples collected at presentation showed significant correlations for IL-6 (r = 0.41; P = .015), IL-8 (r = 0.49; P = .003), IP-10 (r = 0.54; P = .001), MIG (r = 0.46; P = .005), and MCP-1 (r = 0.43; P = .011) (figure 2).

Cytokine and chemokine concentrations at presentation were analyzed according to patient baseline characteristics and clinical outcomes. Older age (≥60 years) was significantly associated with higher IL-6 (P = .019, by Mann-Whitney U test), MIG (P = .017), and MCP-1 (P = .010) concentrations, whereas the presence of major comorbidities was associated with higher IL-6 (P = .018, by Mann-Whitney U test), IL-8 (P = .012), and MIG (P = .043) concentrations. Higher concentrations of virus in NPA samples were also found in these patients (P < .05). Higher IL-6 concentration tended to be associated with the presence of cardiorespiratory complications (P = .069) and had a statistically significant association with prolonged hospitalization (P = .006). A similar trend was observed for the association between MIG concentration and complications (P = .085). A multivariate logistic regression model showed that elevated IL-6 concentration was independently associated with prolonged hospitalization (duration of

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%) of patients (n = 39)</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>18 (46.2)</td>
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<tr>
<td>&lt;60 years</td>
<td>21 (53.8)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (51.3)</td>
</tr>
<tr>
<td>Female</td>
<td>19 (48.7)</td>
</tr>
<tr>
<td>Nursing home resident</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (12.8)</td>
</tr>
<tr>
<td>No</td>
<td>34 (87.2)</td>
</tr>
<tr>
<td>Comorbiditya</td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>22 (56.4)</td>
</tr>
<tr>
<td>Major</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Complicationsb</td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>29 (74.4)</td>
</tr>
<tr>
<td>Respiratory</td>
<td>21 (53.8)</td>
</tr>
<tr>
<td>Supplemental oxygen use</td>
<td>19 (48.7)</td>
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<tr>
<td>Noninvasive ventilatory support</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>Hospital length of stay &gt;5 days</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14 (35.9)</td>
</tr>
<tr>
<td>No</td>
<td>25 (64.1)</td>
</tr>
</tbody>
</table>

**NOTE.** Mean (±SD) values for complete blood count, determined at presentation for all 39 patients, were as follows: WBC count, 7.4 ± 2.6 × 10⁶ cells/L; neutrophil count, 5.8 ± 2.5 × 10⁶ cells/L; lymphocyte count, 0.8 ± 0.4 × 10⁶ cells/L; platelet count, 216 ± 76 × 10⁹ platelets/L; and hemoglobin count, 12.9 ± 1.7 g/dL.

a The major comorbidity category includes congestive heart failure and cerebrovascular, neoplastic, and chronic liver and renal diseases; classification is based on the Pneumonia Patients Outcome Research Team Severity Index scoring system. These patients might also have other coexisting illnesses. The any comorbidity category includes the presence of major comorbidity or other significant medical illnesses, including diabetes, ischemic heart disease, chronic pulmonary disease (e.g., asthma, chronic obstructive pulmonary disease, or bronchiectasis), and neurological diseases [29].

b The any influenza-related complications category includes new events or exacerbation of underlying medical problems [29]. The respiratory complications category includes lower respiratory tract involvement, including a clinical diagnosis of pneumonia, bronchitis, or exacerbation of chronic pulmonary diseases. Other documented complications included acute cardiovascular or cerebrovascular events (n = 4) and dehydration or azotemia, mental dullness, delirium, or syncope (n = 8). Some patients had >1 complication. A significant sputum culture result (positive for Streptococcus pneumoniae) was noted in 1 patient. No other coinfection due to viral or bacterial pathogens was detected by culture.

Table 1. Description of baseline characteristics and clinical outcomes for 39 consecutive patients hospitalized with severe influenza A virus infection.
hospitalization, >5 days), adjusted for age, comorbidity, and initial NPA sample virus concentration (P = .020).

**Signaling molecules.** To examine a possible signaling pathway, differential expression of phospho-p38 MAPK and phospho-ERK molecules in T lymphocytes of 12 patients with acute influenza were compared with those of 14 control subjects. Enhanced phospho-p38 MAPK and suppressed phospho-ERK expressions in CD4 T cells were observed during acute illness (P < .05, by Mann-Whitney U test; figure 3A and 3B). In CD8+ cells, the expression of phospho-ERK was suppressed, but there was no enhanced expression of phospho-p38 MAPK, compared with results for control samples (figure 3C and 3D). Expression of phospho-p38 MAPK in CD4+ Th cells correlated significantly with IP-10 (ρ = 0.78; P = .004) and MCP-1 (ρ = 0.70; P = .016) concentrations in the acute-phase blood samples, and a trend was shown for MIG concentration (ρ = 0.57; P = .066).

**DISCUSSION**

Our results suggest that influenza virus induces inflammatory (e.g., IL-6 and IL-8) and hyper-Th type 1 (Th1) cell immune responses (e.g., IP-10 and MIG) during the early, active viral-replicating phase of the disease. A more intense cytokine response is associated with a higher nasopharyngeal viral load and is detected among “high risk” patients with prolonged, severe illness. It is possible that early viral suppression may attenuate these potentially deleterious cytokine responses.

Our findings are consistent with those of earlier studies of human influenza, which show that local and systemic cytokine responses, such as elevation of IL-6 and IL-8 concentrations, can be detected in vivo and that the intensity of the cytokine responses can be correlated with symptom severity [17–19]. We have further demonstrated that, in severe, complicated influenza, certain chemokines (e.g., IP-10 and MIG) may be important in immunopathogenesis, and aberrant cytokine and chemokine responses are present in patients with advanced age, comorbidity, and extended illnesses. Notably, the cytokine and chemokine profile observed in patients with severe human influenza is similar to that observed in patients with avian influenza (i.e., patients infected with the HSN1 strain). In avian influenza, elevated plasma concentrations of IL-6, IL-8, IP-10, and MIG have also been detected, but the profile is extended to include MCP-1, IL-10, and IFN-γ [9–11]. Moreover, their concentrations are noted to be much higher, which is an observation that is consistent with laboratory study findings that

<table>
<thead>
<tr>
<th>Cytokine or chemokine</th>
<th>Acute-phase samples (median pg/mL, IQR)</th>
<th>Convalescent-phase samples (median pg/mL, IQR)</th>
<th>Reference range, a pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>10.6 (4.2–18.4)</td>
<td>2.9 (1.6–7.0)</td>
<td>&lt;3.1</td>
</tr>
<tr>
<td>IL-8</td>
<td>5.4 (2.5–8.7)</td>
<td>2.1 (0.2–3.5)</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>IP-10</td>
<td>7043.0 (4025.1–12381.1)</td>
<td>1423.6 (931.8–1634.8)</td>
<td>202–1480</td>
</tr>
<tr>
<td>MIG</td>
<td>992.1 (499.1–1992.3)</td>
<td>431.7 (198.4–792.9)</td>
<td>48–482</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>16.0 (13.5–18.6)</td>
<td>15.7 (10.1–16.7)</td>
<td>&lt;15.6</td>
</tr>
<tr>
<td>IL-12</td>
<td>1.8 (1.8–1.9)</td>
<td>1.8 (1.7–1.9)</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.7 (1.6–1.8)</td>
<td>1.7 (1.6–1.8)</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.5 (2.5–6.5)</td>
<td>2.2 (1.9–2.8)</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.4 (1.4–1.9)</td>
<td>1.4 (1.4–1.7)</td>
<td>&lt;3.9</td>
</tr>
<tr>
<td>RANTES</td>
<td>1851.8 (667.4–4774.3)</td>
<td>4742.8 (2767.5–5169.7)</td>
<td>4382–18783</td>
</tr>
<tr>
<td>MCP-1</td>
<td>76.5 (49.5–97.0)</td>
<td>56.6 (41.2–84.8)</td>
<td>10–57</td>
</tr>
</tbody>
</table>

**NOTE.** Plasma concentrations of acute-phase and convalescent-phase samples were compared using Wilcoxon signed-rank test. IP-10, IFN-induced protein 10; IQR, interquartile range; MCP-1, monocyte chemoattractant protein 1; MIG, monokine induced by IFN-γ; RANTES, regulated upon activation, normal T cell–expressed and secreted.

a The reference ranges were derived from the measurement of samples obtained from >100 healthy control subjects [33].

b P < .01.
c P < .001.
d P < .05.

The figure is available in its entirety in the online edition of *Clinical Infectious Diseases.*
Figure 2. Correlations between plasma cytokine and chemokine concentrations measured at presentation (expressed as log₁₀ pg/mL) and initial influenza A virus concentrations in nasopharyngeal aspirate samples (expressed as log₁₀ copies/mL). Spearman’s rank correlation coefficient was used to assess correlations (IL-6, $\rho = 0.41$ and $P = .015$; IL-8, $\rho = 0.49$ and $P = .003$; IFN-induced protein 10 (IP-10), $\rho = 0.54$ and $P = .001$; monokine induced by IFN-γ (MIG), $\rho = 0.46$ and $P = .005$; and monocyte chemoattractant protein 1 (MCP-1), $\rho = 0.43$ and $P = .011$).
Hypercytokinemia in Severe Influenza

The H5N1 strain is more potent with respect to cytokine induction than the H1N1 or H3N2 strains because of different internal gene constellations (e.g., NS1 gene) [12–14]. The hypercytokinemia in avian influenza may have contributed to the high mortality rate associated with this disease (∼50%) [8]. Although the data may not be directly comparable, it seems that the cytokine and chemokine concentrations measured in patients with severe human influenza are, in turn, higher than those reported for previously health individuals with mild, uncomplicated disease (similar flow cytometric assays were used in these studies) [10, 11].

Consistent with earlier reports, we have identified IL-6 as the key proinflammatory cytokine responsible for fever and influenza symptom formation. Elevated IL-6 concentrations have been associated with a higher degree of fever and higher symptom score, as well as certain influenza-related complications, such as encephalopathy [7, 17–19, 21–23]. We have further shown that it is independently associated with prolonged, severe illness. The exact immunopathogenetic mechanisms and symptom correlations of IL-8 (a neutrophil chemoattractant, implicated in the pathogenesis of acute respiratory distress syndrome), IP-10 (a chemoattractant of monocytes and/or macrophage and Th1 cells, a major systemic inflammatory mediator via activation of cell-mediated immunity), and MIG (also indicating activation of Th1 cell pathway) in severe influenza infection require further study [5, 6, 33, 35]. Importantly, we demonstrated a positive correlation between virus replication and hypercytokinemia, suggesting that early initiation of effective antiviral therapy may be able to attenuate these potentially harmful inflammatory responses [11, 19, 20].

This study is, to our knowledge, the first to examine in vivo the activation of intracellular signal transduction pathways in lymphocyte subsets of patients with influenza. It has been shown that phospho-p38 MAPK is involved in the signaling mechanisms that induce cytokine expression and apoptosis in infected cell lines and in animal models of influenza infection [6, 25, 26]. In T cells, it regulates IFN-γ production and the Th1 cell response during early infection [19, 25, 36, 37]. Our findings indicate that hyperactivation of phospho-p38 MAPK is present in the CD4+ Th cells; taken together with its correlation with enhanced expression of certain Th1 cell chemokines (e.g., IP-10, which is inducible by IFN-γ), the results suggest that a hyper-Th1 cell immune response is possibly involved in the pathogenesis of acute severe influenza [10, 36]. The role of phospho-p38 MAPK in regulating cytokine and chemokine expression in vivo in other cell types (e.g., monocytes and/or macrophages and epithelial cells) [26, 27] warrants further investigation. Because the signaling molecule phospho-ERK is responsible for cell proliferation [38], its suppression in Th cells, suppressor T cells, and cytotoxic T cells may explain the moderate lymphopenia commonly observed among pa-

**Figure 3.** Differential expression of signal transduction molecules phospho-p38 mitogen-activated protein kinase (pp38) and phospho-extracellular signal-regulated protein kinase (pERK) in CD4+ T helper cells (A and B) and CD8+ suppressor T cells plus cytotoxic T cells (C and D) in patients with severe influenza (influenza group), compared with healthy control subjects (control group). The Mann-Whitney U test was used to determine statistical significance. MFI, mean fluorescence intensity; *P < .05; **P < .01; ***P < .001.
tients with influenza [11, 39]. Enhanced phospho-p38 MAPK and suppressed phospho-ERK expressions in mononuclear cells have also been detected in severe acute respiratory syndrome, associated with elevated circulating chemokine levels [27]. These findings on signal transduction may provide a biochemical basis to examine a potential strategy of anti-Th1–mediated inflammation in treating severe influenza [37, 40].

Our study is limited by a small sample size and a restricted range of measured cytokines and/or chemokines; it is also limited by the fact that only systemic cytokine responses, as opposed to local responses (e.g., respiratory-tract specific responses), were examined [11, 18]. The inevitable slight delay in sampling time after symptom onset in naturally occurring infections might have affected the detection of certain cytokines and/or chemokines (e.g., TNF-α, IFN-α, and IFN-γ) that are released very early in the course of the illness [18, 19]. Although the virus concentrations in NPA samples are not exact representations of the total body virus burden or virus replication intensity in the lower respiratory tract, they can give a semi-quantitative estimate of the amount of virus shedding in the upper respiratory tract in cases of human influenza infection [11, 18]. Additional studies should include a detailed mechanistic study of signaling pathways and cytokine induction using different strains (e.g., H3N2) of influenza virus and various immune cells (e.g., monocytes and/or macrophages) and epithelial cells; measurement of other chemokines, such as the epithelial neutrophil-activating peptide 78 (a neutrophil chemoattractant), macrophage inflammatory protein 1 α and macrophage inflammatory protein 1 β (monocyte chemoattractants) [41, 42]; measurement of cytokine and chemokine concentrations at local inflammatory sites to correlate disease manifestations [43, 44]; and the effect of early antiviral therapy on cytokine suppression [19, 20]. The novel but controversial approach of anticytokine and antichemokine immunotherapy (using antibodies or antagonists) in treating severe influenza is also of research interest [26, 40, 45, 46].

In conclusion, hypercytokinemia (of proinflammatory and Th1 cytokines) is detected in severe influenza and correlates with clinical illness severity and virus concentration. Hyperactivation of phospho-p38 MAPK signaling pathway (in Th cells) is possibly involved. Early viral suppression may attenuate these potentially deleterious cytokine responses.

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