

# Role of cytokines and chemokines in severe and complicated influenza infections

## Key Messages

1. Exuberant systemic cytokine and chemokine responses are present in naturally occurring influenza infection. These are associated with more severe clinical manifestations, and can be linked to uncontrolled viral replication and signalling molecule hyperactivation.
2. Early antiviral treatment may suppress these harmful cytokine/chemokine responses.

## Introduction

Severe seasonal influenza accounts for more than 226 000 hospital admissions in the United States annually.<sup>1</sup> In Hong Kong it is responsible for 15 to 50 hospital admissions per 10 000 elderly people who may suffer from various complications including pneumonia, bronchitis, exacerbations of chronic pulmonary diseases, and even heart attacks and strokes.<sup>2</sup> The mortality rate in these hospitalised patients is high, ranging from 4 to 29%, but few clinical studies have investigated the immunopathogenesis of severe influenza infection.<sup>3,4</sup> In mild, uncomplicated H1N1 infections (either experimental or naturally occurring), plasma and nasal IL-6 concentrations have been found to be elevated, and to correlate with both viral titres and systemic and respiratory symptoms. Significant releases of IL-8, TNF- $\alpha$ , IFN- $\alpha/\gamma$ , and IL-10 into nasal fluid or peripheral blood have also been detected. Nonetheless, the number of cytokines that have been studied is limited, and the question whether certain chemokines (eg IP-10) play a role in naturally occurring influenza infections remains unanswered.<sup>5,6</sup> Studies on H5N1 (avian influenza) disease have suggested that a 'cytokine storm' (eg IL-6, IL-8, IP-10, MIG, MCP-1) occurs due to uncontrolled viral replication and may be responsible for its clinical manifestations and poor outcomes.<sup>7-11</sup> Hyperactivation of certain signalling molecules such as phospho-p38 mitogen-activated protein kinase (MAPK) has been linked to the production of this 'cytokine storm'. With the imminent threat of another influenza pandemic, studies on the immunopathogenesis of severe influenza enable hypothesis generation for developing novel therapeutic approaches.

In this study, we aimed to detect systemic cytokine and chemokine responses in naturally occurring, severe human influenza infection, and to examine their correlations with clinical disease severity, level of viral replication, and signalling molecule activation. We found an intense pro-inflammatory and Th1 cytokine/chemokine response in severe infection that is associated with uncontrolled viral replication.

## Aims and objectives

1. To study the changes of various cytokines/chemokines among hospitalised acute influenza-infected patients;
2. To compare pre-treatment cytokine/chemokine profiles with their convalescent phase profiles; and
3. To correlate these with age, co-morbidity, symptom severity, complications, and viral loads.

## Methods

### Patients

A prospective observational study on consecutive laboratory-confirmed adult (age  $\geq 18$  years) influenza patients admitted to the Prince of Wales Hospital during a peak flu season in 2006 (1 February to 31 July 2006) was conducted. Patients were diagnosed and managed according to the standard protocol. They were admitted to designated medical wards and placed on droplet precautions when they had developed potentially serious medical conditions, or when the

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Department of Medicine and Therapeutics,  
The Chinese University of Hong Kong,  
Shatin, NT, Hong Kong SAR  
NLS Lee

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Principal applicant and corresponding author:  
Dr Nelson LS Lee  
Department of Medicine and Therapeutics,  
The Chinese University of Hong Kong,  
Shatin, NT, Hong Kong SAR, China  
Tel: (852) 2632 3174  
Fax: (852) 2637 3852  
E-mail: leelsn@cuhk.edu.hk

exacerbation of their chronic underlying illnesses or severe symptoms were considered unmanageable at home.<sup>12</sup> A nasopharyngeal aspirate (NPA) was taken to test for influenza A and B infections using immunofluorescence assays (IFA; the panel also included parainfluenza, respiratory syncytial virus [RSV] and adenovirus), together with blood and sputum cultures to exclude secondary infections. Patients were recruited once their NPA/IFA tested positive for influenza A. After obtaining informed consent, 12 mL ethylenediaminetetraacetic acid venous blood samples (the acute phase samples) were taken from the patients on the same day as antiviral treatment commenced. Patients were followed up 7 to 10 days after the acute symptoms had subsided, and the blood sampling was repeated (convalescent phase samples). There was no medical intervention. The patients were managed and discharged according to their physicians' usual practice. In particular, antiviral treatment choices were not affected by their decision to participate. The institutional review board of the Chinese University of Hong Kong and the Hospital Authority of Hong Kong approved this study.

### ***Virological investigations***

The NPA samples collected were used for immunofluorescence staining, virus isolation and influenza viral RNA quantification. A commercial IFA for influenza A and B (Chemicon International, CA, US) was used to make the initial diagnosis of influenza infection. Influenza virus isolation was conducted using MDCK cells, and cell monolayers were examined daily for any cytopathic effect. After 14 days of incubation, the growth of influenza was examined using haemadsorption, and confirmed by immunofluorescence staining using influenza group-specific antibodies (Chemicon International, CA, US), which identified the isolate as either influenza A or B. Influenza A isolates were further differentiated into H1 and H3 subtypes. To estimate the amount of influenza A RNA in the upper respiratory tract, total RNA was extracted from the supernatants of the NPA specimen using a QIAamp Viral RNA extraction kit (Qiagen, Hilden, Germany). The resulting complementary DNA products (cDNAs) were subjected immediately to a real-time polymerase chain reaction (PCR). The primers used were 5'-AAG ACC AAT CCT GTC ACC TCT GA-3' (forward) and 5'-CAA AGC GTC TAC GCT GCA GTC C-3' (reverse), which amplified a 74-base pair fragment in the M gene of influenza A. These primers were designed to detect influenza A RNA originating from the circulating H1N1 and H3N2 viruses. The real-time PCRs were carried out in a 96-well microtitre plate using ABI7900 (Applied Biosystems).<sup>13</sup>

### ***Measurement of plasma cytokines and chemokines***

The ethylenediaminetetraacetic acid blood samples were immersed in ice and transported immediately for processing. Plasma was separated by centrifugation (2000 xg 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, tumour necrosis factor (TNF)-α; and chemokine

CXCL8/IL-8, monokine induced by IFN-γ (CXCL9/MIG), IFN-γ-inducible protein-10 (CXCL10/IP-10), monocyte chemoattractant protein-1 (CCL2/MCP-1), and regulated upon activation normal T cell-expressed and secreted (CCL5/RANTES) were measured simultaneously using bead-based multiplex flow cytometry with human inflammatory cytokine and chemokine cytometric bead array (CBA) reagents, respectively (four-colour FACSCalibur flow cytometer, BD Biosciences Corp, San Jose [CA], US). The choice of cytokines and chemokines investigated was based on the results of previous studies on seasonal and avian influenza infection. The CBA uses different bead populations with distinct fluorescence intensities that have been coated with capturing antibodies specific for different cytokines or chemokines. After incubation with 50 µL of plasma, the beads that had captured cytokines/chemokines were mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. Fluorescence flow cytometry of the beads provide simultaneous quantification of a panel of cytokines and chemokines. Plasma concentrations of IFN-γ were quantified using an ELISA kit (R&D Systems, MN, USA). The assay sensitivities of IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, TNF-α, CXCL8/IL8, CCL5/RANTES, CCL2/MCP-1, CXCL10/IP-10 and CXCL9/MIG were 7.1, 2.5, 3.3, 3.7, 1.9, 7.2, 0.2, 1.0, 2.7, 2.8, 2.5 pg/mL, respectively, as previously published. The coefficients of variation were all <10%. Their respective reference ranges (RF) were derived from the measurement of more than 100 healthy controls as previously described.<sup>14</sup>

### ***Analysis of intracellular signalling molecules***

Differential activations of selected intracellular signalling molecules, including phospho-p38 MAPK, phospho-extracellular signal-regulated protein kinase (ERK), and pJNK in the T-lymphocytes (both CD4+ and CD8+ cells) were studied in the first 12 patients and compared to 14 age- and sex-matched healthy controls. In brief, PBMC was separated; fluorochrome-conjugated antihuman MAPK or mouse IgG isotopic antibody was added, incubated with the cells, and subjected to flow cytometric analysis using CD4/CD8 cell gating. Results were expressed as mean fluorescence intensity (MFI).<sup>15</sup>

## **Results**

Thirty-nine patients with H1N1 infection were studied. Their mean age was 57±21 years and 56% had underlying medical conditions. Over 70% of patients developed influenza-related complications, which included pneumonia, bronchitis, exacerbation of chronic obstructive pulmonary disease/asthma, acute cardiovascular or cerebrovascular events, encephalopathy and syncope. Nearly half of the patients required supplemental oxygen therapy. Over 35% of these patients had prolonged hospitalisation for more than 5 days. No patient in this cohort died.

Cytokine/chemokine concentrations (pg/mL) in plasma samples obtained during the acute phase were compared to

convalescent phase concentrations. We found significant increases in IL-6 (10.6 [range, 4.2-18.4] vs 2.9 [range, 1.6-7.0]; RF, <3.1), IL-8 (5.4 [range, 2.5-8.7] vs 2.1 [range, 0.2-3.5]; RF, <5.0), IP-10 (7043.0 [range, 4025.1-12381.1] vs 1423.6 [range, 931.8-1634.8]; RF, 202-1480), MIG (992.1 [range, 499.1-1992.3] vs 431.7 [range, 198.4-792.9]; RF, 48-482) and MCP-1 (76.5 [range, 49.5-97.0] vs 56.6 [range, 41.2-84.8]; RF, 10.0-57.0) during the acute illness (overall 2-5 fold increase; Wilcoxon signed-rank test,  $P < 0.01$ ). The highest cytokine/chemokine concentrations were noted on symptom onset days 3 to 4. During the convalescent phase, all these cytokine/chemokine levels dropped, but the RANTES concentration increased (1851.8 [range, 667.4-4774.3] vs 4742.8 [range, 2767.5-5169.7];  $P < 0.05$ ). The acute phase samples were collected  $2.8 \pm 1.2$  days after symptom onset. No significant changes in IFN- $\gamma$ , IL-1 $\beta$ , IL-10, IL-12p70 and TNF- $\alpha$  concentrations were detected.

Viral RNA concentrations in the upper respiratory tract were found to correlate significantly with IL-6 (Spearman's  $\rho = +0.41$ ,  $P = 0.015$ ), IL-8 ( $\rho = +0.49$ ,  $P = 0.003$ ), IP-10 ( $\rho = +0.54$ ,  $P = 0.001$ ), MIG ( $\rho = +0.46$ ,  $P = 0.005$ ) and MCP-1 ( $\rho = +0.43$ ,  $P = 0.011$ ).

Hypercytokinaemia (eg IL-6, IL-8, MIG, MCP-1) occurred in patients who were of advanced age, had major comorbidities, and developed cardiac/respiratory complications (Mann-Whitney  $U$  test, all  $P < 0.05$ ). These patients were also found to have higher viral loads (RNA concentration) in their respiratory tracts ( $P < 0.05$ ). In a multivariate logistic regression analysis, it was found that an elevated IL-6 plasma concentration was independently associated with prolonged hospitalisation of  $>5$  days, adjusted for age, co-morbidity and viral load (odds ratio [OR], 14.4;  $P = 0.020$ ).

Hypercytokinaemia was linked to hyperactivation of intracellular signalling molecules. In CD4+ T-helper cells, the expression of p38-MAPK was enhanced (ie higher MFI) and phospho-ERK was suppressed (Mann-Whitney  $U$  test,  $P < 0.05$ ); in CD8+ T-cells, both phospho-ERK and pJNK were noted to be suppressed, compared with healthy controls (Mann-Whitney  $U$  test,  $P < 0.05$ ). Expression of p38-MAPK was found to be associated with elevated IP-10 ( $\rho = +0.78$ ,  $P = 0.004$ ), MCP-1 ( $\rho = +0.70$ ,  $P = 0.016$ ), and MIG ( $\rho = +0.57$ ,  $P = 0.066$ ) concentrations.

## Discussion

Exuberant systemic cytokine and chemokine responses are present in naturally occurring influenza infection. These are associated with more severe clinical manifestations, and can be linked to uncontrolled viral replication and signalling molecule hyperactivation. It is possible that early antiviral treatment can suppress these harmful cytokine/chemokine responses. Our findings add to the existing knowledge about influenza immunopathogenesis, and enable hypothesis generation for the development of novel

therapeutic approaches against severe influenza infection.

These findings are consistent with earlier *in vitro* and *in vivo* cytokine studies on uncomplicated influenza. In mild human H1N1 infection, local (nasal) and systemic (peripheral blood) cytokine responses, such as elevated IL-6 and IL-8 levels, can be detected, and these correlate with symptom severity.<sup>10</sup> In severe, complicated naturally occurring influenza infection, chemokines such as IP-10, MIG and MCP-1 are detectable and have clinical correlations, along with the established influenza chemokines IL-6 and IL-8. IL-6 is a key pro-inflammatory cytokine responsible for fever and influenza symptom formation.<sup>11</sup> It has been associated with various influenza-related complications, including encephalopathy and cardio-respiratory events. Elevated IL-6 is an independent factor associated with prolonged, severe illnesses in hospitalised patients. The exact immunopathogenetic mechanisms and symptom correlations of IL-8 (a neutrophil chemoattractant, implicated in the pathogenesis of ARDS), IP-10 (a chemoattractant for monocytes/macrophages and Th1 cells, which acts as a major systemic inflammatory mediator by activating cell-mediated immunity) and MIG (an indicator of activation of the Th-1 pathway) in severe human influenza infection are less clear and require further study. The cytokine profile observed in naturally occurring influenza is quite similar to that observed in avian influenza (H5N1) disease. In those cases, elevated plasma concentrations of IL-6, IL-8, IP-10 and MIG are seen, but the profile includes other cytokines/chemokines such as MCP-1, IL-10, TNF- $\alpha$  and IFN- $\alpha/\gamma$ . In addition, systemic concentrations of these cytokines are much higher than those seen in seasonal influenza as reported in this study. The H5N1 virus is known to be a more potent inducer of cytokines than H1N1 or H3N2, which is possibly related to its different internal gene constellation (eg the NS1 gene).<sup>7-9</sup>

Our findings also help to explain the severe symptoms in hospitalised influenza patients.<sup>12</sup> Exuberant cytokine/chemokine responses are present in high-risk patients who are of advanced age and have underlying co-morbid illnesses. Their cytokine levels are much higher than those described for younger patients with uncomplicated influenza. It is likely that impaired host-defence in the high-risk patients have led to more active viral replication (higher viral loads), which in turn induces a more intense cytokine response. Since a positive correlation between virus replication and hypercytokinaemia can be demonstrated, it is possible that early effective suppression of viral replication by antiviral treatment (eg oseltamivir, zanamivir) may result in attenuation of these harmful inflammatory responses.

Our novel finding of *in vivo* activation of intracellular signalling molecules in acute influenza infection may deserve further investigation. It has been shown that p38-MAPK can induce cytokine expression and apoptosis in experimental influenza models. This molecule is activated in T-lymphocytes in naturally occurring infection, and

its intensity of expression correlates with a hyper-Th1 cytokine response. These findings provide a basis for further investigation of novel therapeutic approaches for treating severe influenza, such as through modulation of cytokines and signalling molecules.<sup>5,6</sup>

## Conclusions

Exuberant systemic cytokine and chemokine responses are present in naturally occurring, severe influenza infection, and can be linked to uncontrolled viral replication and signalling molecule hyperactivation. Our findings add to existing knowledge concerning the immunopathogenesis of severe influenza, and enable hypothesis generation for the development of novel therapeutic approaches. This study has broadened our understanding of the immunopathogenesis of severe influenza infection, and has important implications for its clinical management. Hypercytokinaemia occurs in high-risk patients and is related to uncontrolled viral replication. Early, effective viral suppression may result in the attenuation of these harmful cytokine responses and efforts should be made to achieve this goal. Further investigation into the immunopathogenesis of influenza is warranted as this may enable the development of new therapeutic approaches.

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