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ORIGINAL ARTICLE

Temporal changes in cytokine/chemokine profiles and pulmonary involvement in severe acute respiratory syndrome

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Temporal changes in cytokine/chemokine profiles and pulmonary involvement in severe acute respiratory syndrome
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Objective and background: Pathological changes in severe acute respiratory syndrome (SARS) suggest that SARS sequelae are associated with dysregulation of cytokine and chemokine production. To improve understanding of the immuno-pathological processes involved in lung injury associated with SARS, the temporal changes in cytokine/chemokine profiles in the sera of SARS patients were compared with those of patients with community-acquired pneumonia (CAP), according to the degree of lung involvement.
Methods: Serum levels of 11 cytokines and chemokines, in 14 patients with SARS and 24 patients with CAP, were serially checked using a bead-based multiassay system. Sera from 12 healthy subjects were used as normal controls.
Results: The serum levels of interferon-γ-inducible protein-10 (IP-10), IL-2 and IL-6 were significantly elevated during SARS infection. In patients with CAP, but not in those with SARS, the levels of interferon-γ, IL-10, IL-8 and monokine induced by interferon-γ (MIG) were significantly elevated compared with the levels in healthy controls. Among the chemokines/cytokines, IL-6 levels correlated most strongly with radiographic scores ($r = 0.62$). The elevation of IP-10 and IL-2 antedated the development of chest involvement and reached peak levels earlier than the radiographic scores. In contrast, the dynamic changes in IL-6, C-reactive protein and neutrophils occurred synchronously with the changes in radiographic scores. The mean ratio of IL-6 to IL-10 in SARS patients (4.84; range 0.41–21) was significantly higher than that in CAP patients (2.95; range 0.02–10.57) ($P = 0.04$).
Conclusions: The early induction of IP-10 and IL-2, as well as the subsequent over-production of IL-6 and lack of IL-10 production, probably contribute to the main immuno-pathological processes involved in lung injury in SARS. These changes in cytokine/chemokine profile are remarkably different from those observed in CAP patients.

Key words: community-acquired pneumonia, cytokine, severe acute respiratory syndrome.

INTRODUCTION

Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a novel SARS-coronavirus (SARS-CoV).[1] The major clinical features of SARS include fever, dyspnoea, lymphopenia and a rapid progression of pulmonary infiltrates on CXR.[2,3] SARS-related deaths result mainly from pulmonary complications, that is, progressive respiratory failure due to alveolar damage and acute respiratory distress syndrome (ARDS).

Histopathological examination of lung specimens from SARS patients also reveals features of diffuse alveolar damage with marked pulmonary oedema and hyaline membrane formation. Those findings imply a dysregulated immune reaction as a key component of the SARS-induced pulmonary lesions.[4] The

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fact that some patients still manifested lung injury at a time when the viral load was falling also supports the immunological nature of the lung damage.[5]

In order to improve the understanding of the immuno-pathogenesis of SARS, an analysis of the dynamic changes in cytokine/chemokine profiles was undertaken in SARS patients who initially had a normal CXR, but who later progressed to typical manifestations of lung involvement.

METHODS

Patient selection

During the epidemic period of SARS in Taiwan, the total number of SARS patients was 671. Seventy-six patients were diagnosed as probable cases in the National Taiwan University Hospital. Of these, 14 who initially had a normal CXR were enrolled in this study. They had a mean age of 46 years (range 24–87) and nine were female. All except two (both with controlled hypertension for more than 5 years) were previously healthy. All patients met the World Health Organization (WHO) case definition for SARS[6] and had positive real-time polymerase chain reaction assays for the SARS-CoV in clinical specimens, or had positive seroconversion in 28-day convalescent sera.

Serum samples were collected prospectively from the SARS patients, from admission to the 14th treatment day. Serum samples from 24 patients with non-SARS community-acquired pneumonia (CAP), admitted between July and December 2004, were collected in a similar manner.

Sera taken from the patients with SARS and non-SARS CAP were then distributed into four groups, according to disease progression on CXR: admission (A), the day of admission; progressive stage (P), the day lung involvement was observed; worst stage (W), the day maximum lung involvement was observed; and convalescent stage (C), 7–10 days after the worst stage. Twelve serum specimens from healthy adults, aged 24–78 years (mean 53.5), were used as normal control samples (N).

For all the study subjects, excluding healthy controls, IgG antibody to the SARS-CoV was assessed by a standard indirect fluorescence antibody assay.[7] The study was approved by the Institutional Review Board of the National Taiwan University Hospital. Informed consent was obtained from all subjects.

Treatment protocol for SARS

Most of the enrolled patients received a standardized treatment protocol. Oral ribavirin was used soon after the diagnosis of SARS was established, with a loading dose of 2000 mg, followed by 1200 mg per day for 10 days, unless adverse effects developed. Methylprednisolone (2 mg/kg/day for five days) was usually administered in the second week of the disease course, if the patient developed a flare of fever, progression of clinical symptoms (such as dyspnoea or diarrhoea), a surge or resurge in the level of C-reactive protein (CRP), or a rapid deterioration of CXR findings.

Once the patients were intubated and supported by a mechanical ventilator, respiratory care was provided according to the principles for management of ARDS.

Cytokine/chemokine assays

Except for IL-6, which was measured by an enzyme-linked immunosorbent assay (IMMULITE, Diagnostic Products Corporation, Los Angeles, CA, USA), the concentrations of the cytokines, interferon-γ (IFN- γ), tumour necrosis factor-α (TNF- α), IL-5, IL-4, IL-2 and IL-10, as well as the chemokines, monocyte chemoattractant protein-1 (MCP-1), monokine induced by IFN-γ (MIG), IFN-γ-inducible protein-10 (IP-10) and IL-8 were measured using the human Th1/Th2 cytokine and chemokine CBA kits (BD Pharmingen, CA, USA), respectively.[8,9] The assays were performed according to the manufacturer's instructions and the assay sensitivities for the six cytokines and four chemokines were 2.6, 2.6, 2.4, 2.8, 2.8, 7.1, 0.2, 2.7, 2.8 and 2.5 pg/mL, respectively. The assay sensitivity for IL-6 was 2 pg/mL. All measurements were performed in duplicate.

CXR and evaluation

CXRs were obtained daily during treatment. Each lung was visually assessed for areas of involvement of the opacities according to a scoring system of 0–10, where a score of 0 signified normality; score 1, 10% of the total lung area involved; score 2, 20% of the total lung area involved; and so on up to a score of 10. This provided a maximum score of 10 for each lung and a score of 20 for all lung fields (radiographic score).[10] CXR scores were assessed by two chest physicians who were blinded to the patients' clinical data.

CXR scores on the day of admission (stage A), the day of initial lung involvement (stage P), the day of maximum lung involvement (stage W), and 7–10 days after the worst stage (stage C), for each patient, were subjected to statistical analysis.

Statistical analysis

Categorical variables were compared using Fisher's exact test. Differences in continuous variables were analysed using the Wilcoxon rank sum test or analysis of variance (ANOVA). The Pearson correlation coefficient (r) was used to express the correlation between continuous variables, while linear regression was used to estimate correlations between cytokines and radiographic score. The goodness-of-fit was expressed as an r-squared value. All statistical analyses were performed using Stata version 7 software (College Station, TX, USA). Data are presented as mean (range) unless stated otherwise. All reported P-values are two-sided and a P-value < 0.05 was considered to be statistically significant.

RESULTS

Patient characteristics

The characteristics of the 14 SARS patients, 24 CAP patients and 12 healthy controls are summarized in Table 1. For the 14 SARS patients with initial negative CXR, the initial CXR and blood samples were obtained at an average of 1.2 days (range 0–5 days) after the onset of fever. All their CXR began to show evidence of air-space opacities on subsequent follow up after an average of 3.7 days (range 1–8 days). All but one patient recruited for this study received corticosteroid treatment and one died of ARDS.

All the CAP patients had symptoms and signs of pneumonia, air-space opacities on initial CXR and a mean time from onset of symptoms to initial CXR of 1 day (range 0–2 days). The causative agents of CAP were *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Morganella* spp. and *Escherichia coli* in seven (29%), three (13%), two (8%), two (8%), two (8%), one (4%), one (4%) and one (4%) patient, respectively.

Both neutrophil and lymphocyte counts were higher in the CAP patients than in the SARS patients, but corticosteroids were not given to any of the CAP patients. Other clinical characteristics of the two groups were similar.

Serum cytokines/chemokines in SARS in different stages of lung involvement

To check the cytokine/chemokine expression profiles according to the severity of lung involvement, 11 cytokines/chemokines were assayed in serial serum samples taken from patients with SARS and different stages of lung involvement. The levels of IFN-γ, TNF-α, IL-4, IL-10, MCP-1 and IL-8 showed no significant elevation compared with those of normal control samples (Fig. 1). IL-2 increased significantly in all stages. IL-6 and IP-10 were significantly elevated in stages A, P and W ($P < 0.01$), and returned to the normal range in stage C. IL-5 and MIG were slightly elevated only in stage W, but not in stages A, P or C.

Comparison of cytokine/chemokine profiles between SARS and CAP patients

To clarify the specificity of cytokine/chemokine profiles in SARS, 24 CAP patients were selected for the measurements of cytokines/chemokines. The results showed that the level of IL-2 in SARS patients was significantly elevated compared with that in CAP patients, at all time points. In addition, the levels of IP-10 in patients with SARS showed significantly greater increases than those in CAP patients in stages P and W, but not in stage C.

Table 1 Baseline characteristics of SARS patients, CAP patients and healthy control subjects[†]

	SARS patients	CAP patients	Healthy controls	*P*-value
Number	14	24	12	
Mean age, years (range)	46 (24–87)	68 (31–86)	54 (24–78)	0.22
Gender (female/male)	9/5	6/18	5/7	0.06
Combined comorbidity, *n* (%)	2 (14)	14 (58)	—	0.08
Initial manifestation				
Fever, *n* (%)	14 (100)	20 (83)	—	0.28
Cough, *n* (%)	2 (14)	24 (100)	—	<0.01
Dyspnoea, *n* (%)	0 (0)	5 (21)	—	0.14
Myalgia, *n* (%)	9 (64)	2 (8)	—	<0.01
Diarrhoea, *n* (%)	1 (7)	0 (0)	—	0.37
Nausea/vomiting, *n* (%)	1 (7)	0 (0)	—	0.37
Laboratory findings				
Creatinine, mean μmol/L (range)	106.1 (35.4–132.6)	114.9 (53.0–424.3)	—	0.54
Alanine aminotransferase, mean U/L (range)	34.4 (17–123)	40.9 (10–245)	—	0.67
Aspartate aminotransferase, mean U/L (range)	21.5 (8–39)	39.2 (3–186)	—	0.19
WCC, mean $\times 10^9$/L (range)	6.1 (4.1–8.7)	15.8 (9.8–20.2)	—	<0.01
Neutrophils, mean $\times 10^9$/L (range)	4.4 (2.1–6.7)	13.5 (8.5–17.4)	—	<0.01
Lymphocytes, mean $\times 10^9$/L (range)	1.1 (0.6–1.7)	1.3 (0.7–2.4)	—	<0.01
C-reactive protein, mean mg/L (range)	64 (2–127)	75 (4–167)	—	0.55
Clinical course				
Severe lung involvement (>50% of lung), *n* (%)	3 (21)	4 (17)	—	0.43
Death, *n* (%)	1 (7)	0 (0)	—	0.37

[†]Data are presented as median (range).
CAP, community-acquired pneumonia; SARS, severe acute respiratory syndrome; —, no data.

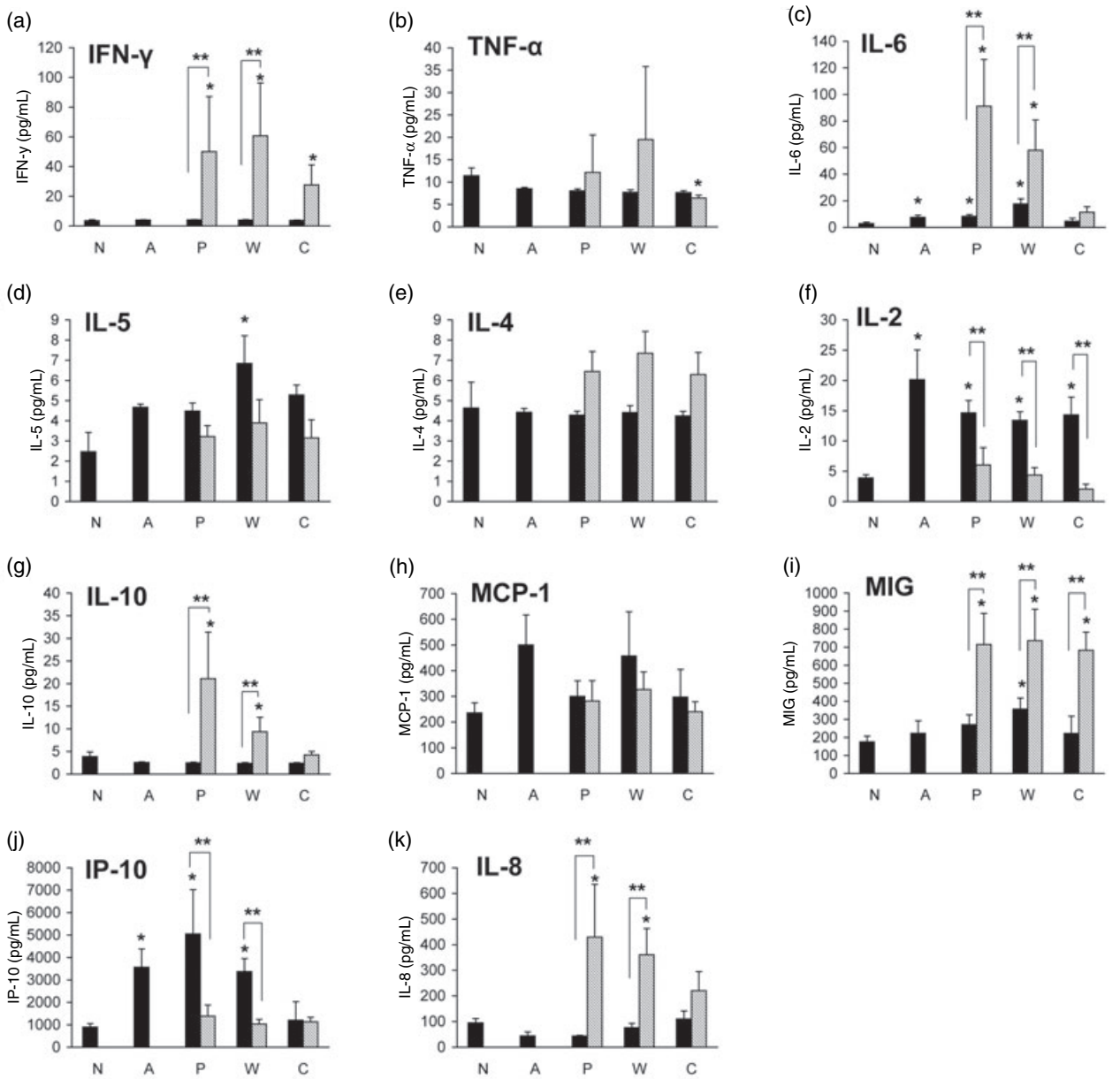

Figure 1 Cytokine/chemokine profiles in the blood of patients with severe acute respiratory syndrome (SARS) and community-acquired pneumonia (CAP). (a–k) Levels of the cytokines interferon (IFN)-, tumour necrosis factor (TNF)-, IL-6, IL-5, IL-4, IL-2, IL-10; and the chemokines monocyte chemoattractant protein (MCP)-1, monokine induced by IFN-γ (MIG), IFN-γ-inducible protein-10 (IP-10) and IL-8 in the blood of patients with SARS or CAP at different stages: A, admission; C, convalescent stage; N, normal control; P, progressive stage; W, worst stage. $^{*}P < 0.05$ compared with normal control group. $^{**}P < 0.05$ SARS (■) versus CAP (▨) groups.

Although the level of IL-6 in patients with SARS was significantly elevated compared with that in normal control samples, it was still much lower than that of patients with CAP. The level of MIG in SARS patients was only slightly elevated in stage W as compared with that of normal control samples, but was much higher in patients with CAP. The levels of IFN-γ, IL-10 and IL-8, although not elevated in patients with SARS, were significantly increased in patients with CAP in stages P and W.

To measure the relationship between pro-inflammatory and anti-inflammatory cytokines, the ratio of IL-6 to IL-10 were calculated. The mean ratio of IL-6 to IL-10 in SARS patients (4.84; range 0.41–21) was significantly higher than that in CAP patients (2.95; range 0.02–10.57) ($P = 0.04$).

Correlation between radiographic score and cytokine/chemokine levels, CRP and hemogram

The correlation between radiographic score and serum levels of cytokine/chemokine, CRP, and neutrophil and lymphocyte counts at all time points

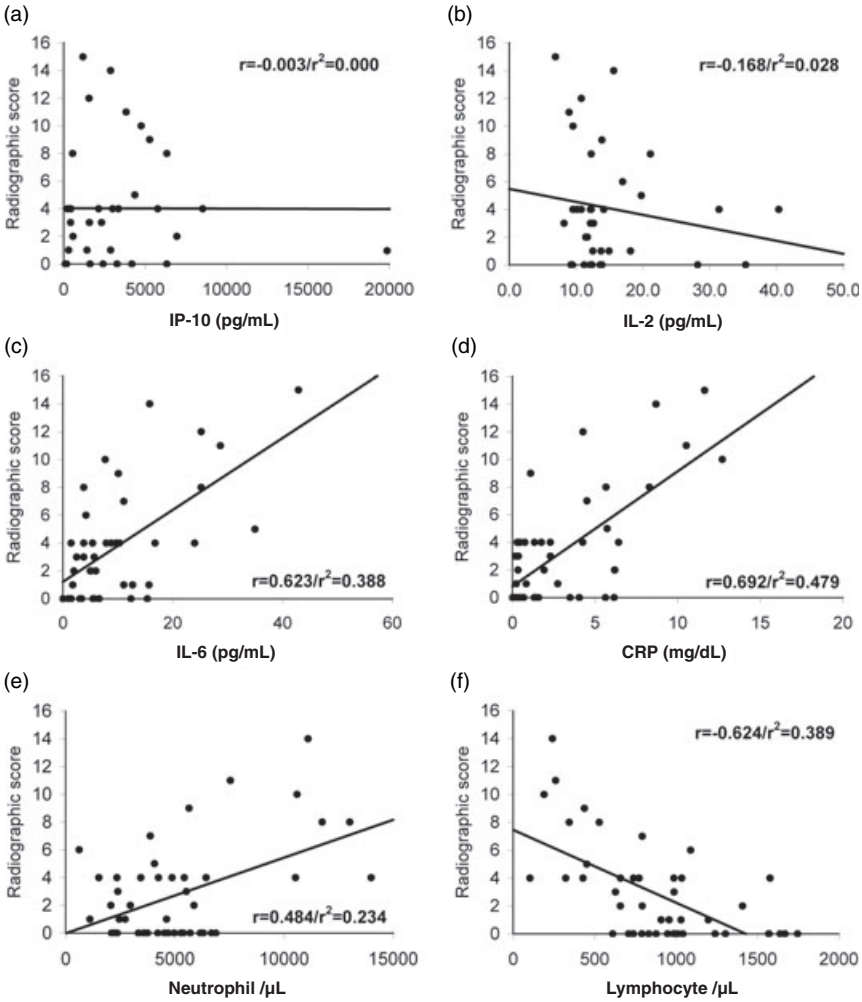

Figure 2 Association between levels of interferon-γ-inducible protein-10 (IP-10) (a), IL-2 (b), IL-6 (c), CRP (d), neutrophil count (e), and lymphocyte count (f), and radiographic score during severe acute respiratory syndrome (SARS) infection. Data from all stages of SARS infection was pooled. Linear regression and correlation were analysed; r = correlation coefficient. CRP, C-reactive protein.

during the SARS infection were analysed (Fig. 2). Among all cytokines/chemokines, IL-6 levels were most positively correlated with radiographic scores ($r = 0.62$) (Fig. 2C). CRP levels were also positively correlated with radiographic scores ($r = 0.69$) (Fig. 2D) and IL-6 levels ($r = 0.64$).

In the hemogram, the neutrophil count showed a positive correlation with radiographic scores ($r = 0.48$). In contrast, the lymphocyte count showed an inverse correlation ($r = -0.62$). The elevation of IP-10 and IL-2 both ante-dated the development of chest involvement and reached their peak levels earlier than the radiographic scores (Fig. 3), which led to poor correlations between IL-10 and IL-2 levels, and radiographic scores. On the other hand, the dynamic changes in IL-6, CRP and neutrophils occurred synchronously with the changes in radiographic scores, while lymphopenia reached its lowest point when the radiographic scores were highest (Fig. 3).

DISCUSSION

Cytokines, particularly chemokines, not only participate in the process of antimicrobial immunity but are also responsible for immuno-pathological injury to host cells, causing major morbidity or even mortality in many respiratory diseases.[11–13] Studying the inflammatory profile in SARS and how it differs from that of other infections, such as non-SARS CAP, may improve our understanding of the immuno-pathological process in SARS. In this study, we investigated 14 SARS patients with initially normal CXR and 24 patients with non-SARS CAP, to clarify the association between temporal changes in cytokine/chemokine profiles and the severity of lung involvement during SARS.

IP-10 and IL-2 are known to play important roles in chemo-attracting and activating monocytes/macrophages,[12–14] which are the predominant inflammatory cell types found in the lungs of patients with SARS.[15] Huang *et al.* found an extremely high serum level of IP-10 in the acute stage of SARS.[5] In a study of 183 patients, Tang *et al.* also found increased IP-10 levels during the first week of SARS, and this was associated with an adverse outcome. Recently, Jiang *et al.*[16] confirmed the expression of IP-10 in pneumocytes, $CD3^+$ T lymphocytes and monocytes/macrophages in the lungs of SARS patients.

In the current study, not only IP-10 but also IL-2 levels were elevated in the early stages (before lung

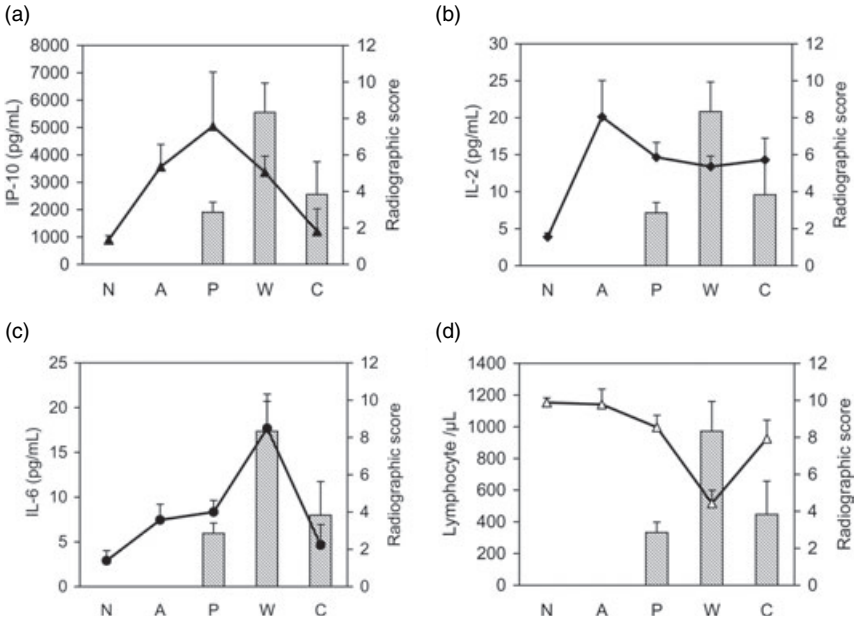

Figure 3 Changes in the levels of interferon-γ-inducible protein-10 (IP-10) (a), IL-2 (b), IL-6 (c) and lymphocyte count (d) in the blood of patients with severe acute respiratory syndrome at different stages: A, admission; C, convalescent stage; N, normal control; P, progressive stage; W, worst stage. (▨) Radiographic score; (—▲—) IP-10; (—◆—) IL-2; (—●—) IL-6; (—△—) lymphocyte.

involvement) of SARS, whereas neither were elevated in patients with non-SARS CAP. These findings suggest that IP-10 and IL-2 might redirect and activate monocytes/macrophages in the lungs and contribute to the pathogenesis of lung injury during SARS infection.[15,17,18]

Similar to the current study, many of the 'classic' cytokines mediating inflammation in acute lung injury,[18] such as TNF-α and IFN-γ, were not reported to be significantly increased during SARS.[19,20] In contrast, circulating concentrations of IL-6 were increased in SARS patients and correlated significantly with the severity of SARS.[19–21] IL-6 has been known to act as a pro-inflammatory cytokine in pulmonary inflammation of various aetiologies, such as CAP,[22] severe pneumonia,[23] bacterial toxin,[24] influenza A infection,[25] radiation-induced lung injury,[26] and hyperoxic lung injury.[27] By dynamic analysis, we showed that after the elevation of IP-10 and IL-2, the progression of lung involvement was better correlated with IL-6 concentration than the levels of other cytokines/chemokines. Therefore, even though IL-6 concentrations were lower in SARS patients than in CAP patients, it still appeared to be an important element of the pathogenesis of lung injury in SARS.

It is interesting to note that IL-8, IFN-γ and IL-10 were not elevated in the SARS patients, whereas they were all increased in CAP patients. As a neutrophil chemokine, IL-8 has been known to correlate with neutrophil numbers in alveolar fluid in ARDS.[18] Elevation of IL-8 has been found in some SARS patients with adverse outcomes.[4] In a study of 23 SARS patients, Jiang *et al.*[16] further found that the elevation of IL-8 was closely associated with super-infection during SARS. Similar elevations of IL-8 in bacterial CAP, but not in SARS infection, was found in our study. The elevation of IL-8 may only indicate a superimposed bacterial infection rather than being a unique indicator of SARS.

IFN-γ, another important Th1 cytokine, establishes innate and adaptive immune reactions against viruses,[28] and has been reported to be elevated in the blood of SARS patients, in two previous studies.[5,20] However, consistent with several other recent studies,[16,21,29] an increase in IFN-γ in the blood of SARS patients was not detected. In a study of dendritic cells, Law *et al.*[29] found that unlike the usual response of dendritic cells to viral infection, antiviral cytokines, including IFN-α, IFN-β, IFN-γ and IL-12p40, were not activated during SARS. Clinically, the SARS-CoV viral load in the respiratory tract peaked at about day 10 after the onset of clinical disease,[30] which is very different from other respiratory viral infections. In common respiratory viral infections, such as respiratory syncytial virus or influenza, peak viral titres are usually reached within 2–3 days after the onset of symptoms.[31] The lack of antiviral cytokines, including interferons, probably allows a longer period for viral proliferation of up to 10 days in SARS-CoV infection.

IL-10, a highly potent anti-inflammatory cytokine, may suppress the production of several pro-inflammatory cytokines during acute lung injury,[18] including CAP.[22] In patients with ARDS, higher concentrations of IL-10 are associated with better survival.[32] Miyaoka *et al.*[33] studied patients after surgery and confirmed that a higher ratio of IL-6 to IL-10 was associated with more severe inflammation. No significant elevation of IL-10 in SARS patients was found, which is consistent with other investigations.[5,16,20] The ratio of IL-6 to IL-10 was also significantly elevated, but more in SARS patients than in CAP patients. Therefore, the lack of IL-10 production during SARS infection might favour a net pro-inflammatory condition, which contributes to the pathogenesis of lung injury and is associated with higher morbidity.

All but one of the patients received corticosteroids during their treatment for SARS. Several longitudinal

observations have suggested that treatment with immuno-suppressive corticosteroids is associated with a reduction in circulating inflammatory cytokine levels in SARS patients.[20,34] However, case-control studies by Zhang *et al*.[19] and Jones *et al*.[21] showed that neither short-course nor long-term treatment with corticosteroids affected the circulating levels of cytokines in SARS patients. Currently, it is difficult to conclude that cytokine profiles are relevant to the use of corticosteroids or the natural process of recovery from illness, independent of any drug treatment.

There were several important limitations in the current study. First, the sample size was small. Second, the patients with CAP were slightly older than the SARS patients, and there were more male patients. Although this difference was not statistically significant, it might also partly contribute to the cytokine/chemokine response during illness. Third, the influence of immuno-suppressive therapy on the profile of cytokines/chemokines in SARS is still uncertain and requires further investigation. Fourth, almost all CAP patients developed pneumonia due to bacterial infection rather than a viral infection. Different pathogens have been known to trigger different cytokine/chemokine profiles in CAP patients. Thus, it might be more informative to compare SARS patients with persons infected with other respiratory viruses rather than bacterial pathogens.

The current findings suggest that the early induction of IP-10 and IL-2, as well as the subsequent overproduction of IL-6 and lack of IL-10 production, probably contribute to the main immuno-pathological processes involved in lung injury in SARS. These findings differ from those observed in subjects with CAP. The early elevation of IP-10 and IL-2 may be early markers of lung injury that could be useful in judging the clinical situation of patients with SARS.

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