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Altered p38 Mitogen-Activated Protein Kinase Expression in Different Leukocytes with Increment of Immunosuppressive Mediators in Patients with Severe Acute Respiratory Syndrome

Chen-Hsiang Lee,∗ Rong-Fu Chen,† Jien-Wei Liu,* Wen-Tien Yeh,† Jen-Chieh Chang,† Po-Mai Liu,† Hock-Liew Eng,‡ Meng-Chih Lin,* and Kuender D. Yang†

Severe acute respiratory syndrome (SARS) has spread to a global pandemic, especially in Asia. The transmission route of SARS has been clarified, but the immunopathogenesis of SARS is unclear. In an age-matched case-control design, we studied immune parameters in 15 SARS patients who were previously healthy. Plasma was harvested for detection of virus load, cytokines, and nitrite/nitrate levels, and blood leukocytes were subjected to flow cytometric analysis of intracellular mitogen-activated protein kinases (MAPKs) in different leukocytes. Patients with SARS had significantly higher IL-8 levels (p = 0.016) in early stage, and higher IL-2 levels (p = 0.039) in late stage than normal controls. Blood TNF-α, IL-6, and IL-10, and nitrite/nitrate levels were not significantly elevated. In contrast, TGF-β and PGE2 levels were significantly elevated in SARS patients. Five of the 15 SARS patients had detectable coronaviruses in blood, but patients with detectable and undetectable viremia had no different profiles of immune mediators. Flow cytometric analysis of MAPKs activation by phospho-p38 and phospho-p44/42 (extracellular signal-regulated kinase) expression showed that augmented p38 activation (p = 0.044) of CD14 monocytes associated with suppressed p38 activation (p = 0.033) of CD8 lymphocytes was found in SARS patients. These results suggest that regulation of TGF-β and PGE2 production and MAPKs activation in different leukocytes may be considered while developing therapeutics for the SARS treatment.


The outbreak of severe acute respiratory syndrome (SARS) outbreak caused by coronavirus originating in Guangdong Province of China has spread to >30 countries, including Taiwan (World Health Organization, http://www.who.int/csr/sars/country2003_07_07/en/). The pathogenesis of SARS is not clear, while the transmission route of SARS has been clarified. The treatment of SARS patients with antivirus therapy or with anti-inflammatory corticosteroids remains highly controversial (1). Studies from Hong Kong favored a combination of antivirus agent with corticosteroids (2, 3), and another report from Canada used a conservative treatment with antivirus therapy (4). It can be hazardous to use corticosteroids without effective antivirus agents in patients with an extensive SARS pneumonitis (5). In contrast, a rapid course of adult respiratory distress syndrome (ARDS) related to possible cytokine storm of SARS infections may require an anti-inflammatory therapy with i.v. corticosteroids. Whether there is directly viral pneumonitis (virus cytotoxic tissue damage) or indirectly immune-mediated tissue damage in SARS infections remains obscure.

A histology of lung necropsy from SARS patients showed that abundant foamy macrophages and multinucleated syncytial cells were demonstrated (3, 6). Thus, cytokine storm has been proposed to be involved in the rapid course of ARDS in SARS patients (2, 3, 6). In contrast, the facts that there is a higher mortality in SARS patients older than 65, and that certain SARS patients tended to have coinfection (1–3) suggest that immunosuppression might also play a role in the pathogenesis. In an attempt to determine whether proinflammatory cytokines or skewed Th (Th1/Th2) cytokines were involved in the pathogenesis of SARS, we collected plasma from SARS patients and controls for measuring proinflammatory cytokines TNF-α, IL-6, and IL-8, as well as Th reaction mediators IL-2, IL-12, IL-10, TGF-β, NO, or PGE2 production. Previous studies with certain viruses including murine coronavirus have shown that inhibition or activation of mitogen-activated protein kinase (MAPK) was involved in induction or suppression of cytokines after virus infection (7, 8). We therefore harvested blood leukocytes in 1% formaldehyde for flow cytometric analysis of intracellular MAPK activation, as demonstrated by phospho-p38 and phospho-extracellular signal-regulated kinase (ERK) expression after plasma collection. The blood cytokines and immunomediators in SARS patients were also related to the intracellular expression of MAPK activation in this study.

Materials and Methods

Hospital outbreak of SARS and the design for this study

On April 26, 2003, an index case who initially presented with flank pain and fever from Taipei was admitted to our hospital at Kaohsiung, southern Taiwan. This patient was not suspected of SARS until April 30. From May
2 to 17, 2003, a cluster of SARS patients including inpatients cases, family caregivers, and health care workers developed probable SARS. We recruited those who were previously healthy adults for this study in a paired case-control design. Once we had recruited 1–3 SARS patients, we included 1–3 age-matched normal adults for blood collection of immune studies. Confirmation of SARS infections was determined by positive RT-PCR detection of coronavirus in acute stage or detectable coronavirus-specific Ab by ELISA in convalescent stage.

Preparation of plasma and leukocytes under a safety procedure

Studies proceeded with a safety procedure. The safety procedure for this study was approved by the Institute Review Board of this hospital. Because the clinical progression of SARS was usually uniform and categorized into two stages, 1) febrile pneumonitis in the first week and 2) shifting pneumonitis, even progressing to ARDS, between the second and third weeks (2–4) and therefore collected whole blood (4 ml) within 7 days and between the second and third weeks of admission. The blood samples were separated into plasma and blood cells by centrifugation at 1500 × g for 15 min. The plasma was harvested for cytokine determination after heat inactivation at 56°C for 15 min, and the blood cells were separated into RBC and white blood cells by dextran (T500, 4.5% sedimentation) (9). The white blood cells were subjected to inactivation of all known potential infectious agents and fixation of leukocytes by 1% formaldehyde. All of the procedures involving the plasma and leukocyte preparation were conducted in a P2 laboratory.

Measurement of blood cytokines and PGE2

Plasma PGE2 and cytokines TNF-α, IL-2, IL-6, IL-8, IL-10, IL-12, and TNFα were measured with an ELISA kits purchased from R&D Systems (Minneapolis, MN). Plasma aliquots at 0.1 ml were used for each individual ELISA, as described earlier (10). To compare the cytokine production in SARS patients with other infectious diseases, we included 15 plasma samples of our patients with dengue hemorrhagic fever, which were used for comparison with SARS patients with other infectious diseases, we included 15 individual ELISA, as described earlier (10). To compare the cytokine production in SARS patients with other infectious diseases, we included 15 plasma samples of our patients with dengue hemorrhagic fever, which were collected in the last year of dengue-2 outbreak in southern Taiwan. We also included 21 plasma samples from bacterial pneumonia with and without ARDS. Seven of the 21 pneumonia patients had ARDS, as defined by bilateral alveolar consolidation greater than two quadrants, oxygen index (PaO2/FiO2) ≥150, and peak end expiratory pressure ≥10 cm H2O (11).

Real-time RT-PCR detection of coronaviruses in blood

We subjected viral RNA extracted from plasma of the controls and patients with SARS to a fluorogenic quantitative RT-PCR detection of total virions in blood, as previously described (12). In brief, 140 μl of plasma was individually added with 560 μl of Qiamp viral RNA extraction solution (Qiagen, Valencia, CA) to cause inactivation and lysis of all potential infectious agents. The viral RNA was further purified by the Qiamp spin column and suspended to 40 μl, per the manufacturer’s recommendation. Each 10 μl RNA sample was subjected to a fluorescent quantitative RT-PCR by the TaqMan method using ABI 7700 quantitative PCR machine (Applied Biosystems, Foster City, CA) for 45 cycles (12). The forward primer, the reverse primer, and the nested fluorescent probe sequence (5′-CCT TCT TTT TGC CCA GCG GAG CCA CAC ATG-3′, and 5′-FAM TCG TGC GTG GAT TTT CTA TTA-3′) were used for detecting coronavirus were, respectively: 5′-CCT TCT TTT TGC CCA GCG GAG CCA CAC ATG-3′, and 5′-FAM TCG TGC GTG GAT TTT CTA TTA-3′ (per the manufacturer). A small amount of plasma (50 μl) was subjected to measurement by the NO analyzer (NO-Analyzer 280; Seivers, Denver, CO) allows the interaction of NO with ozone to elicit chemiluminescence (NO + O3→NO2 + O2; NO2→NO + hν). A small amount of plasma (50 μl) was subjected to measurement by the NO analyzer (NO-Analyzer 280; Seivers, Denver, CO), 10 cm H2O (11).

Blood cytokines and immune mediators

It was found that plasma IL-8 levels were significantly higher in SARS patients than in normal controls in the first week of illness (mean ± SD: 108.5 ± 30.0 vs 73.3 ± 4.4 pg/ml; p = 0.016). The elevated IL-8 levels returned to normal between second and third weeks (Fig. 1A). As shown in Fig. 1C, plasma IL-2 levels in patients with SARS were not significantly higher in the early stage (16.4 ± 3.5 vs 23.1 ± 5.6). However, the IL-2 levels were significantly higher in the second to third week of the illness (16.4 ± 3.5 vs 32.3 ± 5.3; p = 0.039). The elevated IL-8 levels in SARS patients were lower than those in the patients of bacterial pneumonia with ARDS, and those in the patients with dengue hemorrhagic fever (Fig. 2A). Similarly, plasma TNF-α levels in SARS

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SARS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells (×10^6/L)</td>
<td>6.74 ± 0.08</td>
<td>4.36 ± 0.19</td>
<td>0.043</td>
</tr>
<tr>
<td>Lymphocytes (×10^6/L)</td>
<td>2.02 ± 0.12</td>
<td>0.61 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets (×10^12/L)</td>
<td>270.29 ± 4.63</td>
<td>145.47 ± 2.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monocytes (×10^6/L)</td>
<td>0.22 ± 0.04</td>
<td>0.16 ± 0.05</td>
<td>0.391</td>
</tr>
</tbody>
</table>

* Data presented are mean ± SE, and p values were analyzed by Student’s t test.
patients were also lower than those with non-SARS ARDS and those with dengue hemorrhagic fever (DHF) (Fig. 2B). In contrast, plasma TGF-β levels in SARS patients were significantly higher than those with non-SARS ARDS (Fig. 2C). The plasma PGE₂ level in SARS patients was also higher than that in non-SARS ARDS patients, although it did not reach a significant difference (Fig. 2D). The plasma TNF-α (p = 0.305), IL-6 (p = 0.117), IL-10 (p = 0.609), IL-12 (p = 0.403), and NOx (p = 0.459) levels were not significantly different between patients and controls in the first week (Fig. 1 and Table II). TGF-β (p = 0.041) and PGE₂ (p = 0.046) levels were significantly elevated in early stage of patients with SARS than those in age-matched normal controls (Table II). The PGE₂ levels were still significantly elevated, but the IL-12 levels in SARS patients were significantly depressed, in the late stage (second to third week), while plasma TNF-α and IL-10 levels remained at no significant change (Table II). The SARS patients with (n = 9) and without (n = 6) methylprednisolone treatment had no significant differences in IL-8 (p = 0.581), TGF-β (p = 0.802), and PGE₂ (p = 0.921) levels in the late stage of the illness. One patient died of a rapid course of spontaneous pneumothorax and another patient developed pulmonary fibrosis; both had increased TGF-β and PGE₂ levels, but not IL-2, IL-10, IL-12, IL-8, or TNF-α levels. The other 13 patients in this study completely recovered without apparent sequelae.

**Dissociation of virus load with cytokines in blood**

Two plasma samples from each patient with SARS and one plasma sample from normal controls were subjected to a real-time RT-PCR detection of coronavirus in blood. Five of the 15 SARS patients had detectable coronavirus RNA in blood, while none of the 15 controls had detectable coronavirus RNA (Fig. 3A). The coronavirus titers in blood were lower, at a range between 42 and 193 virions/ml, similar to the titer at 190 virions/ml reported by others (16). Patients with and without detectable viremia did not differ in plasma IL-8, TGF-β, or PGE₂ levels (Fig. 3, B–D). The patient who died of spontaneous pneumothorax had no detectable coronavirus in blood on days 3 and 9. Another patient with pulmonary

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**FIGURE 1.** Plasma IL-8, TNF-α, IL-2, and IL-6 levels in patients with SARS. A, The plasma IL-8 levels in controls (ctrl) and SARS patients in early (first week; SARS-E) and late (second to third week; SARS-L) stages. B, The plasma TNF-α levels in controls (ctrl) and SARS patients in early (first week; SARS-E) and late (second to third week; SARS-L) stages. C, The plasma IL-2 levels in controls (ctrl) and SARS patients in early (first week; SARS-E) and late (second to third week; SARS-L) stages. D, The plasma IL-6 levels in controls (ctrl) and SARS patients in early (first week; SARS-E) and late (second to third week; SARS-L) stages. The plasma was collected from 15 age-matched controls (ctrl) and 15 patients with SARS. Data presented are mean ± SE, and p values indicated were analyzed by Student’s t test.
fibrosis also had no detectable virus on days 3 and 11. This suggests that the viremia in SARS infections may not be correlated to clinical severity.

Altered p38 activation in different leukocytes
Using formaldehyde-fixed peripheral blood leukocytes, we first measured the phosphorylated p38 MAPK levels in total leukocytes from patients with SARS and age-matched normal controls in early stage. As shown in a pilot study (Fig. 4B), it was found that SARS patients had an increase in intracellular phospho-p38 level. Results calculated from 15 paired experiments showed that the intracellular phospho-p38 levels in total leukocytes were significantly higher in patients than in controls (Fig. 4B). Further studies showed that CD14-positive monocytes were the leukocytes in SARS patients showing an increase in phospho-p38, but not phospho-p44/42 ERK expression (Fig. 5A). CD4-positive T cells from SARS patients appeared to have a suppressed intracellular phospho-ERK level, but it did not reach a significant difference (Fig. 5B). CD8-positive T cells from SARS patients did, however, have a significantly lower intracellular phospho-p38 level in early stage (Fig. 5C). The phospho-p38 expression in CD8 cells remained significantly suppressed in 2–3 wk after admission, while those in CD14 and CD4 cells no longer had significant increase or decrease of phospho-p38 expression.

Discussion
There are two different pathogeneses frequently described in emerging viral infections. One is direct virus injury, and the other is immune-mediated pathogenesis. A 100% detectable viremia associated with impaired humoral response has been related to fatal outcomes of Ebola infections (17, 18). A heavy viral load with sepsis-like syndrome is found in young infants with enteroviral infections (19). In contrast, immune-mediated enhancement of dengue infections has been described in patients with dengue hemorrhagic fever (20). In this study, we found that patients with SARS tended to have normal to mild elevated TNF-α, IL-6, and IL-8 levels. The blood cytokines in SARS patients are much lower than those in other infections with systemic illness such as bacterial pneumonia with ARDS or patients with dengue hemorrhagic fever. The IL-8 levels observed in our SARS patients were ~6-fold lower than those reported in septic patients described by Headley et al. (21). This suggests that proinflammatory cytokine storm is unlikely involved in the pathogenesis of SARS infections. In contrast, an early elevation of immunosuppressive mediators
PGE₂ and TGF-β associated with later elevation of IL-2 levels may partly explain why SARS patients tended to have a longer clinical course and coinfections (2, 22). This inference is, however, hampered by the limitation of blood cytokine level measurements in determining local or organ-specific immune response pattern.

Patients with SARS usually have a prolonged virus shedding in throat, sputum, and feces (2, 16). The virus load in sputum is much higher than those in other specimens. In a real-time RT-PCR analysis of virus load in three patients, Drosten et al. (16) showed that only 1 of the 3 patients had detectable viruses in blood with 10⁶ times lower than those in sputum. Correlation of coronaviruses in blood to clinical outcomes has not been clarified. In Ebola infections, a detectable viremia with impaired humoral reaction is correlated to fatal outcomes (17, 18). In the present study, we found that 5 of the 15 patients had detectable blood coronavirus RNA. Patients with and without detectable viremia had no different profiles of blood immune mediators, suggesting that viremia in SARS infections may not be the trigger to raise altered immune reaction in blood. Viral replication or altered immune reaction in target tissue may be responsible for the elevation of immunosuppressive mediators in the circulation of SARS patients.

Patients with SARS usually have a rapid progression of pneumonia (2–4). Approximately one-third of the SARS patients developed ARDS, one-tenth of the patients succumbed to death, and one-tenth of patients revealed pulmonary fibrosis (2–4). Histological examinations of lung necropsy from SARS patients have demonstrated infiltration of inflammatory cells associated with foamy macrophages, multinuclear syncytial cells, and occasional hemophagocytic features (3, 6). This has raised the possibility of immunopathological damage of lung tissues. Results from this study showed that an augmented p38, but not p44/42 ERK, MAPK activation in CD14 cells was associated with elevated IL-8 levels in SARS patients. It is limited to directly infer the p38 activation of CD14 monocytes responsible for elevated blood IL-8 levels.

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**Table II. Cytokine profiles in plasma of patients with SARS and controls studied**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Controls</th>
<th>SARS Patients Early Stage</th>
<th>SARS Patients Late Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12, pg/ml</td>
<td>106.5 ± 15.9*</td>
<td>77.3 ± 18.6</td>
<td>27.8 ± 15.4*</td>
</tr>
<tr>
<td>NOx, μM</td>
<td>26.7 ± 7.5</td>
<td>36.5 ± 5.4</td>
<td>ND</td>
</tr>
<tr>
<td>TGF-β, pg/ml</td>
<td>8,485.2 ± 824.9†</td>
<td>14,221.6 ± 2,076.9†</td>
<td>10,433.4 ± 1,413.9</td>
</tr>
<tr>
<td>PGE₂, pg/ml</td>
<td>1,288.7 ± 94.5‡</td>
<td>1,965.6 ± 246.7‡</td>
<td>2,170.2 ± 228.7‡</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
<td>3.0 ± 3.9</td>
<td>5.3 ± 8.1</td>
<td>4.1 ± 1.7</td>
</tr>
</tbody>
</table>

* Data presented are mean ± SE. Symbols *, †, ‡, and §, respectively, indicate p values 0.023, 0.041, 0.046, and 0.003 between both groups, as tested by Student’s t test.

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**FIGURE 3.** Immune mediators in SARS patients with and without a real-time RT-PCR detectable coronavirus RNA in plasma. A, The coronavirus RNA titers in plasma from control (ctrl) and SARS patients. B, Plasma IL-8 levels in SARS patients with and without detectable coronavirus RNA. C, Plasma TGF-β levels in SARS patients with and without detectable coronavirus RNA. D, Plasma PGE₂ levels in SARS patients with and without detectable coronavirus RNA.
without simultaneously measuring intracellular IL-8 and phospho-p38 levels in CD14 monocytes. Results from this study, however, suggest that altered leukocyte p38 activation may contribute to abnormal blood cytokine profile in SARS patients. This is similar to a study with murine coronaviruses, showing that murine coronaviruses could activate p38 and c-jun kinases, but not p44/42.
ERK, that are responsible for IL-6 induction (7). Yao et al. (8) reported that hepatitis C core protein could inhibit ERK activation in T cells, resulting in lower IL-2 induction. In our study, we did not find a significant inhibition of ERK activation in SARS infections, but found a slower increase of IL-2 levels in SARS infections. The slower increase of IL-2 production may not be related to ERK activation, but possibly related to altered p38 activation in different leukocytes from SARS patients. Thus, further studies are needed to explore whether increase of p38 activation in monocytes, but decrease of p38 activation in CD8 lymphocytes from SARS patients is really related to increase of immunosuppressive mediators or virus replication in the lung tissues.

Currently, many efforts are now ongoing to develop a vaccine and anti-SARS medication. Another, faster strategy for the SARS treatment is to expose the immune response to the SARS infection and target the altered immunity. The treatment of SARS patients with steroid remains controversial. The fact that SARS patients had elevated immunosuppressive TGF-β and PGE2, mediators, but not proinflammatory cytokines TNF-α, IL-6, IL-8, and IL-10 in the early stage (first week), associated with later elevated IL-2 levels in SARS patients, suggests that administration of steroid in the early stage may not be suitable, but can be considered in the late stage (second to third week). Based on our study showing discordant p38 MAPK activation in different leukocyte populations and elevated circulating TGF-β and PGE2 levels, it is postulated that regulation of TGF-β and PGE2 production and p38 MAPK activation may be considered while developing therapeutics for the SARS treatment.

Acknowledgments
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References