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

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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

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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-$\alpha$, IL-6, and IL-10, and nitrite/nitrate levels were not significantly elevated. In contrast, TGF-$\beta$ and PGE$_2$ 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-$\beta$ and PGE$_2$ production and MAPKs activation in different leukocytes may be considered while developing therapeutics for the SARS treatment. The Journal of Immunology, 2004, 172: 7841–7847.

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periments, 1% formaldehyde-fixed leukocytes were subjected to cell permeabilization by methanol at 1/4 (v/v) for 30 min after washing twice in PBS. The permeated leukocytes in 0.1-mL aliquots (1 × 10^6 cells/ml) were subjected to dual staining of cell surface molecules by PE-conjugated mouse anti-human CD4, CD8, or CD14 Abs (BD PharMingen, Franklin Lakes, CA), and intracellular signal molecules by rabbit anti-human phospho-p38 or phospho-p44/42 (ERK) Abs (Cell Signaling Technology, Beverly, MA) for 30 min. This was followed by recognition with FITC-conjugated goat anti-rabbit Ig Abs for another 30-min staining. After washing twice with PBS, the reactions were suspended in 0.3 ml of PBS for flow cytometric analysis.

Statistics

Data of cytokine measurement are presented with mean ± SE and statistically analyzed by Student’s t test. Expression of MAPK activation was presented with mean intensities of intracellular phospho-p38 and phospho-ERK levels. The mean fluorescent intensities of intracellular phospho-p38 and phospho-ERK levels between patient and control groups were analyzed by Student’s t test.

Results

Clinical features of the subjects studied

Fifteen SARS patients aged 23–45 and 15 normal controls aged 29–41 were studied. The 15 SARS patients, who were previously healthy, all had typical SARS symptoms/signs, showing fever (>38°C) and varying extent of pneumonia on chest radiography. Patients initially often presented leukopenia (mean 4,360 cells/μm), thrombocytopenia (mean 145,470/μl), and profound lymphopenia (mean 610 cells/μm). Monocytes were not significantly different between SARS patients and controls (Table I). Normal or elevated lactate dehydrogenase and creatine phosphokinase were noted. These patients were treated under a protocol with initial institution of ribavirin 400 mg/m^2/day in the first 7 days after a loading dose of 2 g. These patients were allowed to receive steroid (methylprednisolone, 1 mg/kg/day) after 7 days of admission while there was an exacerbation of pneumonitis. The initial blood samples for immune studies from all these patients were collected between 3 and 7 days of admission, whereas 9 of the 15 patients started receiving methylprednisolone (1 mg/kg/day) with and without pulse therapy (500 mg every 12 h for 2 days) while exacerbated pneumonitis or ARDS occurred.

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>Compartment</th>
<th>Controls</th>
<th>SARS</th>
<th>P</th>
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<tbody>
<tr>
<td>White blood cells (× 10^9/L)</td>
<td>6.74 ± 0.08</td>
<td>4.36 ± 0.19</td>
<td>0.043</td>
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<tr>
<td>Lymphocytes (× 10^9/L)</td>
<td>2.02 ± 0.12</td>
<td>0.61 ± 0.02</td>
<td>&lt;0.001</td>
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<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 (× 10^9/L)</td>
<td>0.22 ± 0.04</td>
<td>0.16 ± 0.05</td>
<td>0.391</td>
</tr>
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</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

![Figure 1](http://www.jimmunol.org/)

**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. 4A), 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 pneumonitis (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

![FIGURE 3](http://www.jimmunol.org/)

**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.

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

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Controls</th>
<th>Early Stage</th>
<th>Late Stage</th>
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</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.
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.

![Figure 4](https://via.placeholder.com/150.png)

**FIGURE 4.** Flow cytometric analysis of phospho-p38 expression in the early stage of peripheral blood leukocytes from SARS patients and controls (ctrl). A, Intracellular phospho-p38 expression in total leukocytes from a representative case-control experiment. B, Intracellular phospho-p38 levels in total leukocytes summarized from 15 paired case-control experiments.

![Figure 5](https://via.placeholder.com/150.png)

**FIGURE 5.** Intracellular phospho-p38 and phospho-ERK expression in the early stage of CD14-, CD4-, and CD8-positive leukocytes. A, Intracellular phospho-p38 and phospho-ERK levels in CD14-positive cells summarized from 15 paired case-control experiments. B, Intracellular phospho-p38 and phospho-ERK levels in CD4 lymphocytes summarized from 15 paired case-control experiments. C, Intracellular phospho-p38 and phospho-ERK levels in CD8 lymphocytes summarized from 15 paired case-control experiments. Results were presented with mean ± SE and analyzed by Student’s t test.
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