行政院國家科學委員會專題研究計畫 成果報告

（總計畫與子計畫一）SARS CoV 感染引起的細胞激素風暴以及肺部細胞的病變

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中文摘要

嚴重急性呼吸道症候群(SARS)是由新的冠狀病毒(SARS-CoV)感染引起的疾病，它已經嚴重地威脅到國人的健康與生活。SARS 病人的致死率高達 10-15%，主要受影響的器官是肺臟，在病毒急性感染上，宿主組織的傷害可以是病毒直接的傷害或免疫反應引起的。病毒的複製引起的細胞病變，在感染早期主要是病毒複製引起的。但在感染後期，當適應性的免疫系統啟動後，病毒的清除通常伴隨著免疫發炎反應，這些免疫反應難免会造成宿主細胞的傷害，病毒量越多，免疫反應越強，宿主細胞的傷害越大。以 SARS-CoV 感染引起肺部的病變為例，在感染早期肺部細胞膜呈透明，有水腫現象，之後有淋巴細胞浸潤，肺泡有肺上皮細胞脫落，肺上皮細胞形成空泡或融合成巨大細胞，顯示肺部細胞受到刺激，有活化及死亡的現象。我們之前的 SARS 整合型計劃研究發現有一個”干擾素-γ的細胞激素風暴”存在於 SARS-CoV 感染早期，SARS 病患急性期的血清中有高量的 IFN-γ, IL-18, TGF-β, IL-6, 但沒有 TNF-α, IL-2, IL-4, IL-10, IL-13 or TNFRI, 另外趨化激素 IP-10, MCP-1, MIG 和 IL-8 也很高，這指出干擾素-γ相關的細胞激素風暴確實發生。本來宿主對抗病毒感染會產生干擾素等細胞激素是一種正常的生理反應，我們以前研究登革病毒感染引起的登革出血熱/登革休克徵候群，或腸病毒 71 造成的肺部水腫，也都發現有干擾素-γ的產生，但 SARS 的干擾素-γ細胞激素風暴和登革病毒或腸病毒 71 的細胞激素風暴有二點主要不同，第一是 SARS 病患的干擾素-γ平均量是登革病患或腸病毒 71 病患的 3 至 4 倍，因為干擾素-γ刺激產生的 IP-10, MIG, MCP-1 也跟著很高。第二是登革病患或腸病毒 71 病患中，干擾素-γ和 IL-10 是同時存在的，所以在身體內，IL-10 會抑制干擾素-γ的作用，因此發炎反應不會持續進行，但在 SARS 病患，干擾素-γ和 IL-10 是呈反比關係，有干擾素-γ者沒有 IL-10，因此干擾素-γ不受 IL-10 的抑制，這可能解釋 SARS 的發炎會持續進行，不易受到抑制。

關鍵詞：干擾素-γ細胞激素風暴；細胞凋亡；Fas；Th1 細胞。

Abstract

Severe acute respiratory syndrome (SARS) caused by SARS-associated coronavirus (SARS-CoV) is a new emerging disease that has affected many countries including Taiwan. The lung is a major target organ, and the mortality of SARS is around 10 – 15%. In the previous SARS program project, we have found that IFN-γ cytokine storm and autoantibody...
generation might play two major roles in the continuing development of lung inflammation at early and late stage post SARS-CoV infection, respectively. There is an IFN-γ cytokine storm in the early phase of SARS-CoV infection. High levels of IFN-γ, IL-18, TGF-β and IL-6, but not of TNF-α, IL-2, IL-4, IL-10, IL-13 or TNFRI, in the acute phase sera of SARS patients. The chemokines IP-10, MCP-1, MIG and IL-8 were also elevated. The cytokine profile in SARS is distinct from that in dengue virus-induced dengue hemorrhagic fever or Enterovirus 71-induced pulmonary edema. Interferon is the host response to virus infection with a balanced and controlled manner. But a strong biased IFN-γ cytokine production (3-4 fold higher and extreme polarization of IFN-γ over IL-10) is unique to SARS.

**Keywords**: IFN-γ cytokine storm; Apoptosis; Fas; Th1

**Introduction**

Severe acute respiratory syndrome (SARS) is a new emerging disease that has affected many countries including Taiwan. A novel virus, the SARS-associated coronavirus (SARS-CoV), has been identified as the causal agent (1-5). A tri-phasic progression for the clinical progression of SARS was reported. Week 1 was characterized by fever, myalgia, and other systemic symptoms that are largely related to the effect of viral replication and cytolysis. As the disease progressed into week 2, the patients frequently had recurrence of fever, onset of diarrhea, and oxygen desaturation due to lung damage. The specific antibodies began to be produced, and correlated with falls in viral load. Severe clinical worsening also occurred at this time. The lung damage is proposed to be related to immunopathological damage as a result of an overexuberant host response, rather than uncontrolled viral replication. In the third phase, some of the patients would begin to develop acute respiratory distress syndrome and require ventilatory support. Nosocomial infection will inevitably occur during this phase of end-organ damage and severe lymphopenia.

The laboratory findings of SARS were lymphopenia, thrombocytopenia, and elevated lactate dehydrogenase and creatine kinase levels. Several factors including advanced age, male sex, a high peak lactate dehydrogenase level, a high peak creatine kinase level, and a high initial absolute neutrophils count were significant predictive factors for intense care unit admission and death. The lung damage included hyaline-membrane formation, interstitial mononuclear inflammatory infiltrates, and desquamation of pneumocytes in alveolar spaces. A focal intraalveolar hemorrhage, necrotic inflammatory debris in small airways, and organizing pneumonia were also found. The mortality of SARS is reported to be around 10 – 15%. Acute viral infection may produce damage to host cells by cytolysis or immunopathological damage. In the early stage, cytolysis is accompanied by viral amplification, such that antiviral drugs may be important in treatment. In the later stage,
when adaptive immune response is mounted, viral clearance can be accompanied by severe inflammatory damage, especially with high viral burden.

How the lung is damaged is not clearly understood. The intensive inflammation developed in the lung will generate cytokines to activate lung epithelial cells to undergo pathological changes (6). Is there a “cytokine storm” that is involved in the pathological changes of lung? In this study, we reported that an “IFN-γ-related cytokine and chemokine storm” is indeed present in the SARS patient and its level was associated with clinical manifestation.

Materials and Methods

Patients.
From March 14, 2003 to August 15, 2003, a total of 346 cases were defined as probable SARS cases based on the medical records reviewed retrospectively plus laboratory data and epidemiological data by the SARS Advisory Committee in CDC-Taiwan and SARS expert committees in National Health Insurance Bureau-Taiwan. Among these patients, 104 samples of acute phase sera or convalescent sera were analyzed for their serum cytokine levels. Epidemiological characteristic and laboratory data of 88 RT-PCR positive cases were examined. The epidemiological characteristics of age and gender and clinical information such as symptoms, underlying diseases, outcomes including death and hospital length-of-stay, as well as laboratory findings of hematological, biochemical, and arterial blood gas data were collected from medical chart records, information of disease investigation, web-based data in our reporting system, and laboratories in CDC-Taiwan.

Analysis of SARS CoV infection.
SARS CoV analysis was done primarily in the CDC-Taiwan Central Laboratory. SARS CoV RT-PCR was used to define the infection. The primers were used according to the CDC-US recommendation (4). The ELISA and immunofluorescence assay were done for serological identification of SARS CoV infection. The acute and convalescent sera were tested in parallel for SARS CoV. A neutralization test was also performed. A combination of ELISA, immunofluorescence and neutralization test was used to define the antibody to SARS CoV. The cases that had serum antibody against SARS CoV were classified as Ab positive group while those who had no sera antibody were classified as Ab negative group. The 51 patients in the Ab(-) group were those that die early with no subsequent serum to collect or elders that had weak immune response. The 37 patients in the Ab(+) group were younger with low mortality, the later serum collected demonstrated his or her SARS CoV infection.

Quantitation of cytokine level.
The cytokines levels were determined by either BD Human Th1/Th2 Cytokine or Chemokine Bead Array (CBA) Kit. The BD Human Th1/Th2 Cytokine CBA Kit (BD PharMingen, San Diego, CA) was used to measure IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ protein levels by flow cytometry in a particle-based immunoassay. This kit allowed simultaneous measurement of 6 cytokines from 50 µl of sample. The assays were performed according to the instructions of the manufacturer. The limits of detection of these immunoassays were 2.6 pg/ml of IL-2, 2.6 pg/ml of IL-4, 2.4 pg/ml of IL-5, 2.8 pg/ml of IL-10, 2.8 pg/ml of TNF-α, and 7.1 pg/ml of IFN-γ. The BD Human Chemokine CBA Kit was used to measure IP-10, MIG, MCP-1 and IL-8 protein levels by flow cytometry in a particle-based immunoassay. This kit allowed simultaneous measurement of 5 chemokines from 50 µl of sample. The limits of detection of these immunoassays were 0.2 pg/ml of IL-8, 1.0 pg/ml of RANTES, 2.5 pg/ml of MIG, 2.7 pg/ml of MCP-1 and 2.8 pg/ml of IP-10. The sTNFRI, TGF-β and TNF-α were detected with ELISA kits from Biosource (Camarillo, CA). The detection limits were 50 pg/ml of sTNFRI, 15.6 pg/ml of TGF-β and 1.7 pg/ml of TNF-α. The IL-18 and IL-13 were detected with ELISA kits from MBL Medical & Biological Laboratories Co., (Nagoya, Japan) and R&D Systems Inc. (Minneapolis, MN), respectively. The detection limits were 12.5 pg/ml of IL-18 and 32 pg/ml of IL-13.

Statistical analysis.
We compared cytokine, clinical and laboratory data between antibody positive and antibody negative group or death and survival group by student’s t-test with software of SigmaPlot 8.0 (SPSS Inc., Chicago, IL). P<0.05 was taken to be significant.

Results
The cytokines and chemokines levels are highly elevated in SARS patients. Using the CBA Th1/Th2 cytokines, chemokines kits and ELISA, fourteen cytokines or chemokines, including IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, TGF-β, IP-10, MCP-1, MIG, RANTES and IL-8, as well as TNFRI, were analyzed. As shown in Fig. 1, in a total 88 RT-PCR-confirmed SARS patients, high levels of IFN-γ, IL-18, TGF-β and IL-6 were found in the sera of SARS patients. The chemokines IP-10, MCP-1, MIG and IL-8 were also elevated. The mean values of IFN-γ, IL-18, TGF-β, IL-6, IP-10, MCP-1, MIG and IL-8 were 456.1 pg/ml, 638.6 pg/ml, 768.9 pg/ml, 245.7 pg/ml, 6775.3 pg/ml, 755.7 pg/ml, 1229.8 pg/ml and 325.6 pg/ml, respectively. The serum levels of cytokines varied a great deal between different individuals, but they were highly elevated in the acute phase post SARS CoV infection, and were significantly different from normal healthy controls. All of these cytokines had returned to normal levels at the convalescent phase (30 days or later post disease onset) (Table 1). In contrast, cytokines TNF-α, IL-2, IL-4, IL-10, IL-13 or TNFRI were very low or undetectable. Among the detected cytokines, IFN-γ and its related
chemokines were especially prominent. Only 18% (16/88) of patients had no detectable IFN-γ in the serum, while 50% of the patients had IFN-γ level higher than 500 pg/ml, and 20% higher than 1000 pg/ml. The IFN-γ-stimulated chemokines such as IP-10, MCP-1 and MIG were also highly elevated.

The Th1/Th2 cytokines profile of SARS was then compared with that of other viral diseases, namely enterovirus 71 (EV71)-induced pulmonary edema (PE) and dengue virus (DV)-induced dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). The amount of IFN-γ observed in the SARS patients was four to six fold-higher than that of PE or DHF/DSS (7-8). The mean values of IFN-γ in PE and DHF/DSS were 68 pg/ml and 111 pg/ml, respectively (Fig. 2a). Surprisingly, IL-10 level was very low as 82% (72/88) of patient had serum IL-10 level lower than 20 pg/ml. This is in contrast to PE and DHF whereas the mean concentration of IL-10 in PE and DHF were 123.6 pg/ml and 81.7 pg/ml, respectively. The multiple cytokines were detected simultaneously by the CBA kit, therefore, the Th1-prone IFN-γ of each individual was plotted to his/her Th2-prone IL-10. A reciprocal relationship between IFN-γ and IL-10 was found for all individuals (Fig. 2f). The IL-10-reciprocal relationship was also found for IL-18, MCP-1, IP-10 and MIG (data not shown). This pattern was different from that of EV 71-induced PE or DV-induced DHF/DSS. In these two diseases, a mix of IFN-γ and IL-10 is present simultaneously. While, with regard to other cytokines such as IL-6, IL-18 and IL-8, there were all present in SARS, PE and DHF (Fig. 2b,2d,2e). But for IL-13, only 8% (5/66) of SARS patients had detectable level higher than detection limit, 32 pg/ml (Fig. 2c). On the contrary, IL-13 was highly elevated in PE patients, and it plays an important role in the pulmonary edema post EV 71 infection (7).

Comparison of cytokines in Ab(-) and Ab(+) groups. To understand the cytokines profile in the developmental process of SARS post SARS CoV infection, the 88 RT-PCR-confirmed cases were divided into antibody negative and antibody positive groups. As shown in Table 2, the mean age of 51 patients in Ab(-) group was significantly higher than that of 37 patients in Ab(+) group. A larger percentage of males than of females were found in the Ab(-) group. On the other hand, a larger percentage of females than males were found in the Ab(+) group. The mortality was also significantly higher in the Ab(-) than in the Ab(+) group. Duration of stay in hospital reflects the mortality rate, therefore, it was lower in the Ab(-) than in the Ab(+) group. Laboratory data on BUN, WBC and CBC were not different between these two groups. IL-6, IL-18, IL-10, TGF-β, IP-10, MIG, MCP-1 and IL-8 were also not significantly different between these two groups: they were all highly elevated. The only difference was the IFN-γ level: the serum IFN-γ level in the Ab(+) group was significantly higher than that in the Ab(-) group. To understand the kinetic changes of these cytokines in the early stage of acute infection, the levels of IFN-γ, IP-10, MCP-1, MIG, IL-18, IL-8, IL-6 and TGF-β in Ab(-)
group were plotted against the days post fever onset (Fig. 3). IFN-γ and its related chemokines MCP-1, MIG and IP-10 were already highly elevated at early days. IFN-γ, MIG, IP-10 and MCP-1 seemed to appear the earliest, and peaked at days 1 – 4 post fever onset. IL-8 and IL-18 increased later, and peaked at days 4 – 6 post fever onset. IL-6 and TGF-β also appeared later. To further delineate the relationship between IFN-γ and its related cytokines, the linear regression between IFN-γ or IL-18 and IP-10, MCP-1 or MIG were analyzed. A good association was found for IL-18 and IP-10, MCP-1 or MIG. The correlation coefficient and r² for IL-18 and IP-10, MCP-1 or MIG was 0.568/0.344, 0.688/0.473, and 0.745/0.555, respectively (Fig. 4). The association is better for IL-18 than for IFN-γ. On the contrary, a reciprocal relationship existed between IL-10 and IFN-γ and other related chemokines because no or low IL-10 was produced. It seems that IL-18, IFN-γ, IP-10, MCP-1, and MIG are activated together during the early phase of SARS CoV acute infection.

The association of IFN-γ, IL-18, TGF-β, IL-6, IP-10, MCP-1, MIG and IL-8 with clinical manifestation of SARS. Since SARS CoV infection can be fatal, and intensive inflammatory IFN-γ response is involved in the disease process, we compared the cytokines between death and survival cases of SARS patients. As shown in Table 3, the mean age of death group was older than that of survival group. The death group also had higher BUN or AST value than the survival group. The SARS CoV-infected patients with underlying diseases were more susceptible to death. Their cytokines IL-18, IP-10, MIG and MCP-1 levels were significantly higher than in the survival group. IL-8 seemed also higher in the death group than in the survival group, but the p value (0.091) was not statistically significant. IP-10, MIG and MCP-1 are chemokines for activated T cells while IL-8 is a chemokine for neutrophils. This suggests that the extensive inflammation is probably responsible for the inflammatory response in the lung of SARS death patients. For the survival group, when MCP-1 and IFN-γ were plotted to the circulating lymphocytes count and monocytes count of the SARS patients, an inverse association was found. For MCP-1 and lymphocyte and monocyte counts, the correlation coefficient and r² were −0.438/0.192 and −0.378/0.143, respectively (Fig. 5). The inverse association was also found for IFN-γ and circulating lymphocyte counts (−0.366/0.134). However, the pattern was different for neutrophils, as a positive association occurred between MCP-1 and circulating neutrophils count (0.432/0.145). As for other parameters, a positive association existed between TGF-β and the duration of stay in hospital in the survival group (Fig. 5e). TGF-β was only detected in 48 % of the patients, and it appeared late post infection. But when generated, its serum level was positively associated with the duration of stay in hospital for the survival group (correlation coefficient, 0.539/ r², 0.291). Based on these data, we concluded that there was an interferon-γ-related cytokine storm after SARS CoV infection, and these cytokines are probably involved in the immunological damage of the host.
**Discussion**

During the SARS outbreak in Taiwan, all the sera from either suspected or probable cases were sent to CDC-Taiwan officially for final diagnosis of SARS CoV infection. Therefore, in this study, the sera analyzed were the first-time point collected after hospitalization, and only the RT-PCR-confirmed cases were compared. This is a cross-section study, as only the first-time point before treatment in acute phase stage was analyzed. The sera collected at the convalescent phase were also determined for their cytokine levels, but all of them had returned to basal levels. We did not have sequential serum samples to understand the kinetic changes of cytokines in individual disease progression. But when the patients were pooled and plotted against the day post fever onset, we found a high elevation of IFN-γ and its related cytokine or chemokine. The serum levels of IL-18, IFN-γ, IP-10, MCP-1, MIG and IL-8 were already very high even at the day of fever onset. The strong bias production toward IFN-γ is different from EV71-induced PE and DV-induced DHF/DSS: a reciprocal relationship existed for individual IFN-γ and IL-10. The over-production of inflammatory IFN-γ and chemokines might be resulting from the lack of IL-10-mediated down-regulation of the immune responses to SARS CoV infection.

IFN-γ is an inflammatory cytokine, and possesses many biological activities (9-10). It can enhance the MHC expression, activate macrophage function, stimulate chemokine production, induce apoptosis, arrest cell cycle, and enhance Fas expression. IFN-γ is primarily produced by NK cells, Th1 cells and macrophages. Its production can be up-regulated by IL-12 and/or IL-18. We found that the chemokines IP-10, MCP-1 and MIG were highly elevated in the acute stage of SARS patients. A strong association existed between IL-18 and IP-10, MCP-1 or MIG. The detection of another chemokine, RANTES, was out of range for the CBA kit, but IFN-γ can stimulate lung epithelial cell line to produce these chemokines including RANTES (unpublished observation). Therefore, RANTES is supposed to also be elevated in SARS infection. These chemokines can recruit activated T cells into the lung. There were several reports on the cytokine levels in SARS patients: proinflammatory cytokines TNF-α, IL-1 and IL-6 were increased in acute stage of SARS CoV infection (11-12). In our study, neither TNF-α nor TNFRI were detectable. This might related to the timing of the samples collected. Lung pathological study has shown intense lymphocytic infiltration in SARS patients (6). IFN-γ is already highly induced at the early Ab(-) stage, it was even higher in the late Ab(+) stage. Therefore, we proposed that the bias IFN-γ and its related chemokine production might be responsible for the abnormal inflammatory response in SARS patients. An IFN-γ-related cytokine storm was induced post SARS CoV infection.
The antibody response to SARS CoV antigen was delayed after SARS CoV infection. It is not detectable until two to three weeks later (5). The Ab(-) group represents the early stage of acute SARS CoV infection as shown by early timing of sera collected after fever onset in Fig. 3. The elders with weak immune response were also included in this category. With this antibody distinction, we found that factors of age, gender, mortality, duration of stay-in-hospital were significantly different between these two groups. Older males had high mortality while young females seemed to have strong immune response and higher IFN-\( \gamma \) production. This is consistent with the risk factor analysis between survival and death groups. The IP-10, MIG and MCP-1 levels were higher in death than in survival groups. It seems that male, older patients who have higher chemokine production or with underlying diseases were more susceptible to death post SARS CoV infection. A severe acute respiratory syndrome in lung was caused by intense chemokine-induced activated T cell-mediated inflammation and might be fatal. IL-8-mediated neutrophil inflammation is probably also involved.

For the surviving SARS patients, the inversed relationship between MCP-1 or IFN-\( \gamma \) and lymphocyte and monocyte count is interesting. Lymphopenia is common in SARS patients. How lymphopenia occur is not clearly understood. IFN-\( \gamma \) was reported to induce apoptosis of activated T cells (13-14). Two possibilities are reasoned: sequestration into lung of \( \beta \)-chemokine-recruited lymphocytes and IFN-\( \gamma \)-induced apoptosis. But for neutrophils, a positive association was found for MCP-1 and neutrophils count. The neutrophils \( \alpha \)-chemokine IL-8 was also elevated, once the neutrophils are recruited into the lung, they will enhance more lung damage.

TGF-\( \beta \) is found to be increased after SARS CoV infection, 48% (32/66) of patients having detectable serum TGF-\( \beta \) at the late stage. The TGF-\( \beta \) level is not significantly different between death and survival group. But when this TGF-\( \beta \) level was plotted to the clinical data of the patient, it was interesting to find a positive association between TGF-\( \beta \) amount and his or her duration of stay-in-hospital for the individuals who survived. Fibrosis is a sequela post SARS CoV infection in some individual. TGF-\( \beta \) can induce proliferation of fibroblasts (15-16), therefore, high production of TGF-\( \beta \) might be involved in the development of fibrosis.

Dexamethasone and intravenous immunoglobulin were used to treat SARS patients with some beneficial effect, although their effects have been debated recently (17). Dexamethasone is known to inhibit the cytokine production and delayed chemokine-recruited inflammation while the immunoglobulin can modulate the cytokine over-action and inhibit the lymphocyte or macrophage activation. If the IFN-\( \gamma \) cytokine storm is induced and is responsible for the immunological damage of the host, it is reasoned that their beneficial
effects were probable due to the interference with the IFN-γ cytokine storm. It was reported that IFN-γ alone and in combination with activation of the Fas pathway induced apoptosis in A549 lung epithelial cells, and this IFN-γ and IFN-γ plus anti-Fas-induced apoptosis could be blocked by dexamethasone (18).

Self evaluation

This manuscript has been accepted to published in Journal of Medical Virology. This is the report to describe the IFN-γ cytokine storm in SARS.