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 病毒及相關蛋白對單核細胞和樹突細胞分泌發炎性細胞激素和趨化激素的影響

計畫類別：整合型計畫
計畫編號：NSC93-2751-B-002-004-Y
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執行單位：國立臺灣大學醫學院臨床醫學研究所

計畫主持人：江伯倫

計畫參與人員：林郁里，王麗潔，彭仲民

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處理方式：本計畫可公開查詢

中　華　民　國 94 年 9 月 23 日

附件一

行政院國家科學委員會補助專題研究計畫 ☒ 成 果 報 告 □期中進度報告

（計畫名稱）

嚴重急性呼吸道症候群的免疫致病機轉(第二年)-(總計畫與子計畫一)SARS 病毒及相關蛋白對單核細胞和數突細胞分泌發炎性細胞激素和趨化激素的影響

計畫類別：□ 個別型計畫　☒ 整合型計畫
計畫編號：NSC 93－2751－B －002－004－Y
執行期間：　93 年　7 月　1 日至　94 年　6 月 30 日

計畫主持人：江伯倫
共同主持人：無
計畫參與人員：林郁里, 王麗潔, 彭仲民

成果報告類型(依經費核定清單規定繳交)：□精簡報告　☒完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：台大醫學院

中　華　民　國　94 年　9 月　23 日

研究計畫成果報告

Abstract

Objective: To explore the immunopathogenesis of severe acute respiratory distress syndrome (SARS) and the mechanism of milder disease in young children than in adults.

Method: The SARS-associated coronavirus (SARS-CoV) viral proteins were prepared from Baculovirus expression system. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult donors by Ficoll-Hypaque density gradient centrifugation. $CD14^+$ monocytes were purified from PBMCs through MACS column. Then the monocytes were cultured with IL-4 and GM-CSF to yield dendritic cells. The SARS-CoV S, N, E, M viral protein were cutltured with monocytes or dendritic cells for 24~48 hrs. The supernatants were measured for cytokine and chemokine levels by ELISA. Besides, the pcDNA-S, N, E, M and viral protein were used to stimulate A549 cell line for NF-kB activation detected by luciferase reporter assay.

Result: The SARS-CoV E protein (500 ng/mL) may induce the secretion of proinflammatory cytokines (IL-1, IL-6, TNF-α) and chemokine of MCP-1 in human monocytes. The SARS-CoV N protein might also induce IL-6 and MCP-1 secretion. Dendritic cells secreted IL-10 and IL-12 cytokines under S protein (1000 ng/mL) stimulation. The pcDNA-S and pcDNA-N (0.2 μg/mL) or N protein (1 μg/mL) may induce NF-κB activation in A549 cells.

Conclusion: The SARS-CoV E protein may induce proinflammatory cytokines and chemokines secretion of moncytes and S protein may induce the release of IL-10 and IL-12 in dendritic cells from healthy adults. Through these cytokines and chemokines, the immune system may be activated and lead to the cytokine storm and the immunopathological damage.

Key words: SARS, dendritic cells, monocyte, NF-kB, A549 cells

Contents

Introduction

Since November 2002, an outbreak of the mysterious illness called severe acute respiratory syndrome (SARS) was noted in Foshan city of Guangdong Province. In February 2003, a physician from Guangdong Province became ill while staying in a Hotel in Hong Kong. The hotel guests became infected and transported the disease to Vietnam, Singapore, Canada, Ireland and the United States. Today, it was reported in 27 Countries including Taiwan[1].

The SARS patients presented with fever (100%), chills (74%), cough (62%), myalgia (54%), malaise (50%), dyspnea (20%) and diarrhea (10%).[2] The symptoms aggravated in the second week and nearly 40% of the patients developed respiratory failure that required assisted ventilation.[3] The mortality rate was reported as 6.5%~7%.[4-5] The specimen from patients was cultured and a new virus called SARS-associated Coronavirus (SARS-CoV) was isolated. The SARS-CoV is a member of the Coronaviridae family of enveloped, positive –stranded RNA viruses.[6] The 27-32 kb genomes of coronaviruses encode 23 putative proteins, including four major structural proteins; nucleocapsid (N), spike (S), membrane (M), and small envelope (E).[6] Guillen et al have identified three regions on the SARS CoV spike glycoprotein with membrane-interacting capabilities, which supports their direct role in SARS CoV-mediated membrane fusion.[7]

The new virus targeted mainly the lower respiratory tract. In the post-mortem lung biopsy, a giant-cell infiltrate was seen with a pronounced increase in macrophages in the alveoli and the interstitium of the lung.[8] Besides, the consistent clinical progression, shifting radiological infiltrates, and an inverted V viral-load profile suggest that worsening in week 2 is unrelated to uncontrolled viral replication but may be related to immunopathological damage.[4]

Although the high morbidity and mortality of SARS in adults, there was rare mortality reported in the children. The report from Hong Kong pointed out that the symptoms of SARS in younger children were milder and the clinical course was not as aggressive as adult. Besides, Kwang et al believed that SARS in children can be a mild and self-limiting disease.[9] Therefore, the aim of the project is to design the experiment to see the differences of immunological responses to SARS-CoV protein in healthy children and adults. We hope that the result could explain the reason of milder disease in younger children and the immunological pathogenesis of SARS.

Materials and Methods

1. Patients and Samples

Twenty healthy children of 5~15 years old and twenty healthy adults of 30~40 years old were enrolled. The informed consents were obtained from all studied subjects. Blood samplings were done for experiment. Besides, cord bloods with informed consents were also drew for study. In addition, monocytes or dendritic cells will be isolated from individual of different age and stimulated with the SARSCoV and related proteins.

2. Isolation and cultue of human monocytes and dendritic cells

① Isolation of mononuclear cells

Mononuclear cells are isolated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation (400×g for 20 min at RT). The mononuclear cells layer is washed two times with sterile Hank's solution.

② Isolation of CD 14^+ monocytes from peripheral mononuclear cells

Add 15 λ of anti-CD14 conjugated magnetic microbeads and 85 λ buffer per 10^7 PBMC for 30 minutes at 4^oC. Use MACS column to purify monocytes.

③ Dendritic cells preparation

Generate DCs by culturing monocytes in RPMI medium supplemented with 10% FCS with IL-4 200 U/mL and GM-CSF 800 U/mL in the concentration of 10^6 cells/mL for 5 days (37^oC/5% CO_2).

3. SARS-CoV protein preparation

Use Baculovirus expression vector for SARS-CoV protein expression. The pBlueBac4.5/V5-His vector were recombinated with SARS-CoV S, N, E, M DNA in insect cells respectively. After selection of recombinated strain, amplification, expression, and secretion of viral protein were done according to manufacturer's guide. The purification of viral protein was done by Ni^{2+} column.

4. Monocytes stimulation

Purified monocytes (10^6 cells/mL) are cultured with S, N, E, M protein or LPS 100 ng/mL (positive control) or RPMI, BSA (negative control) for 24 hrs (37^oC/5% CO_2). The supernatant is measured by ELISA for IL-1, IL-6, IL-8, TNF-α, MCP-1.

5. Dendritic cells stimulation

① Maturation of DC

DCs are stimulated by addition of S protein or LPS 100 ng/mL (positive control) or RPMI (negative control) in the presence of cytokines (IL-4 200 U/mL + GM-CSF 800 U/mL) for 24~48 hrs (37^oC/5% CO_2).

② Cytokine detection

The supernatants are measured by ELISA for IL-10, IL-12 p70 and IL-12 p40.

6. Phenotypic analysis of cultured dendritic cells

To further characterize the surface markers of dendritic cells, antibodies against MHC class II, CD14, CD11c, B7-1, B7-2, CD40 and OX40L will be used to analyze the surface molecules expressed on the cultured dendritic cells. The cells are washed and resuspended in 0.5 ml of PBS with 0.1% sodium azide and subjected to FMF analysis. A total of 10,000 cells are counted and the frequency and mean density of each cell surface marker were determined using appropriate software (FACScan, Becton Dickinson, Mountain View, CA). Controls are cells suspended in medium.

7. Cytokine secretion

Culture supernatants are collected at 24, 48 and 72 hours following co-culture of 2 x 10^5 monocytes or BM-DC cells with the different stimuli. Cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-a are determined by Quantikine M ELISA Kit (R&D Systems, MN, USA).

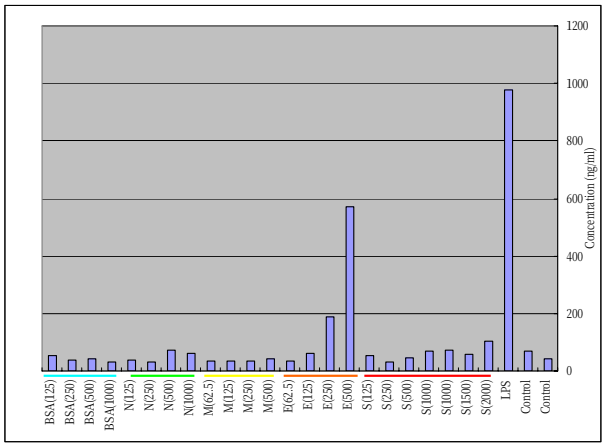
8. NF-kB-dependent reporter assays

Transfect the epithelial cell line (A549) with 3XκB-luciferase reporter plasmid (0.2 μg/mL) and pcDNA-V, pcDNA-S, pcDNA-N, pcDNA-E, pcDNA-M (0.2 μg/mL) respectively. Use the luciferase reporter assay to check NF-κB activity. Monocytes or dendritic cell were plated in 35-mm dishes. On the following day, the cells were transfected with the indicated expression vectors using Lipofectamine (Promega). The reporter plasmid, 3xkB-L (kindly provided by Dr. Nakano, Juntendo University), has three repeats of the NF-kB site upstream of a minimal thymidine kinase promoter and a luciferase gene in pGL-2 vector (Promega). After 24 h, the cells were harvested in PBS and lysed in a luciferase lysis buffer. The lysates were assayed for luciferase activity using a luminometer.

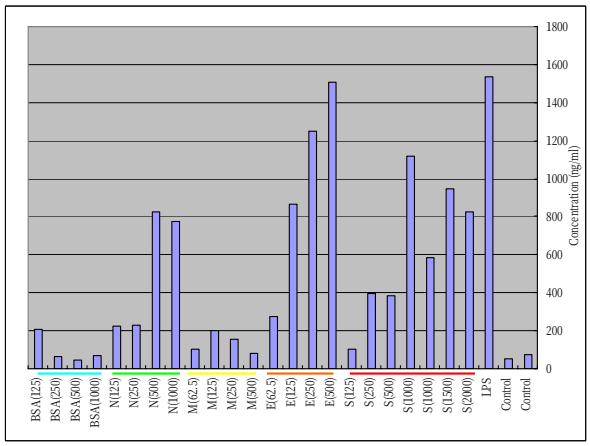
Results

1. Effects of SARS-CoV-related proteins on IL-1β, IL-6, IL-8, TNF-α, MCP-1, IL-12 p40 and IL-12 p70 secretion in human monocyte

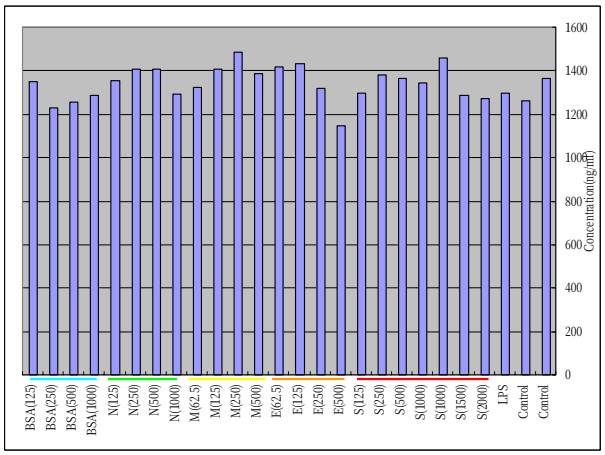
IL-1 β



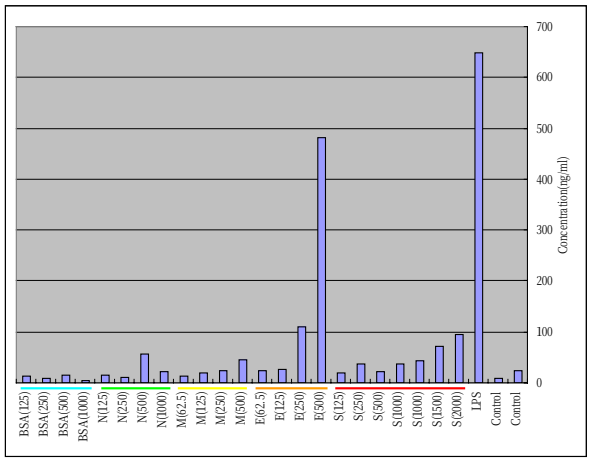
IL-6



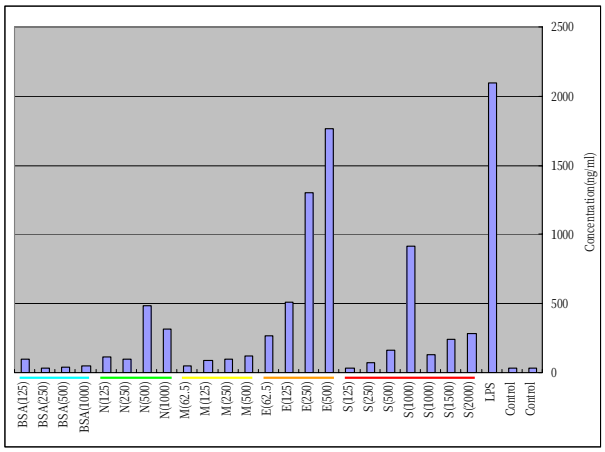
IL-8



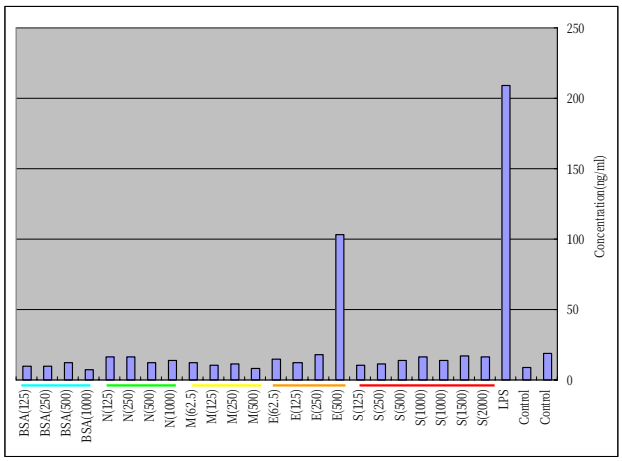
TNF-α



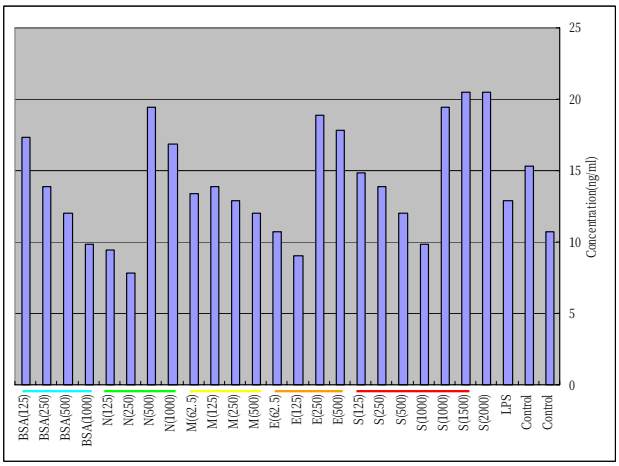
MCP-1



IL-12 p40

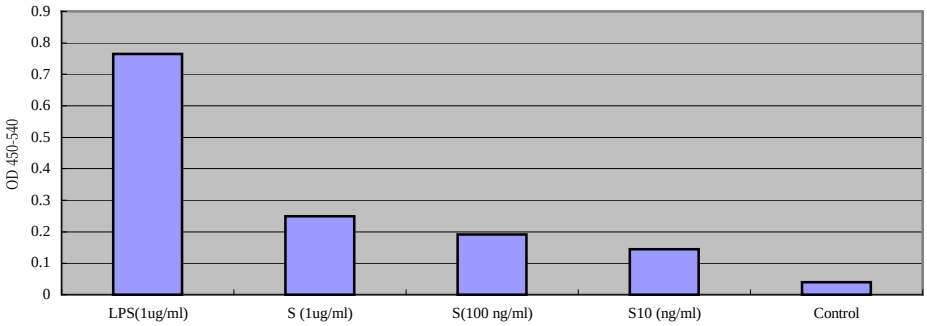


IL-12 p70

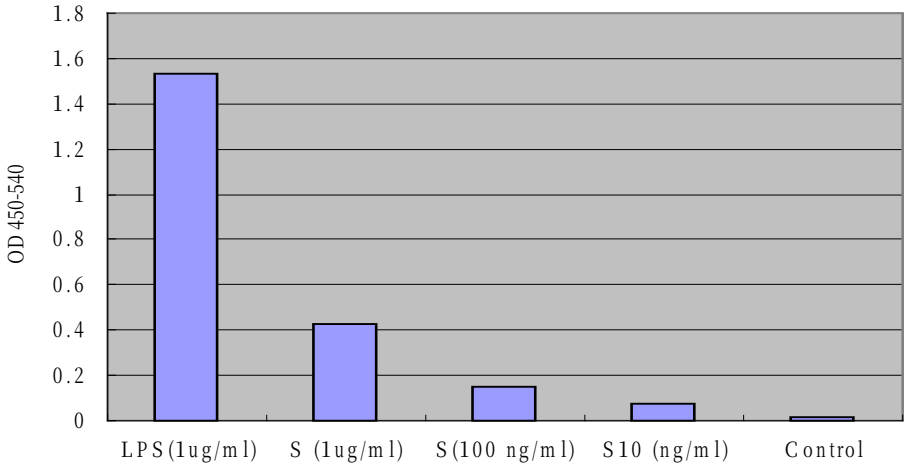


2. Effects of SARS-CoV S protein on IL-10, IL-12 p40, and IL-12 p70 secretion in human dendritic cell

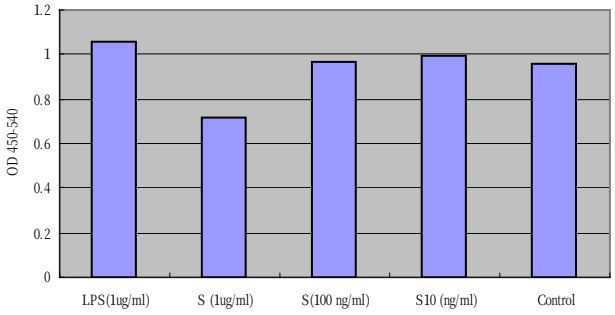
IL-10



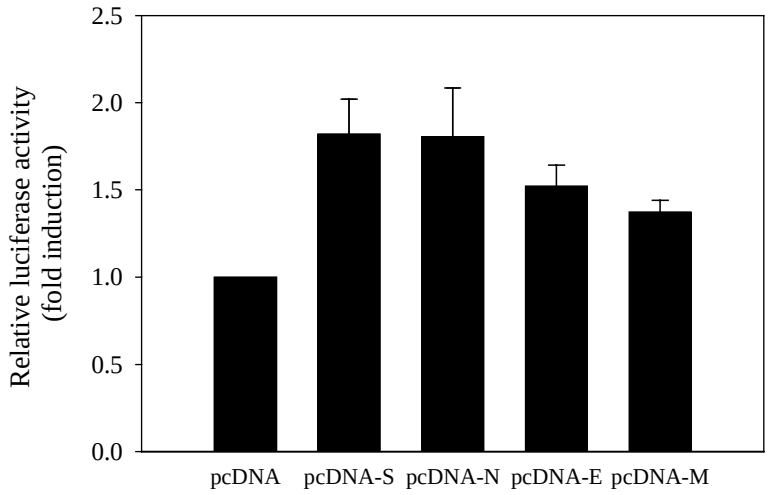
IL-12 p40



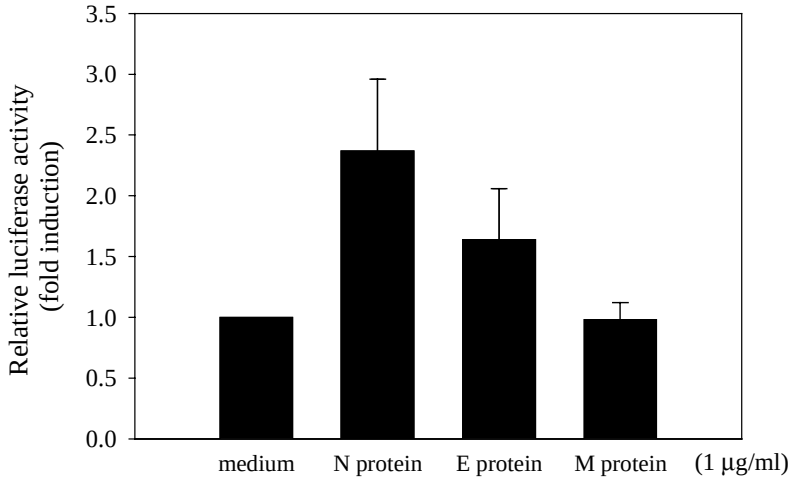
IL-12 p70



3. Effects of pcDNA-S, pcDNA-N, pcDNA-E, and pcDNAM on NF-kB activation in A549 cells



4. Effects of SARS-CoV-related proteins on NF-kB activation in A549 cells



Discussion

Since the immunopathological damage was related to the worsening of the clinical course[4], several centers have measured the cytokine levels in SARS patients. National Cheng Kung University discovered that an interferon-gamma-related cytokine storm including IFN-gamma, IL-18, MCP-1, MIG, and IP-10 was induced post SARS coronavirus infection, and this cytokine storm might be involved in the immunopathological damage in SARS patients.[10] Southern Medical University in Guangzhou found that induction of IP-10 is a critical event in the initiation of immune-mediated acute lung injury and lymphocyte apoptosis during the development of SARS. The prompt elevation of IL-6, IL-8 and MCP-1 is a sign of superinfection indicating a high risk of death.[11]

Our study showed that SARS-CoV E protein prepared from Baculovirus expression system may induce the secretion of proinflammatory cytokines (IL-1, IL-6, TNF-α) and chemokine of MCP-1 in human monocytes from healthy adults. The SARS-CoV N protein might also induce IL-6 and MCP-1 secretion. Therefore, the monocytes may launch the immune system after encounter SARS virus through these cytokines and chemokines. Besides, the dendritic cells cultured in vitro from healthy adults secreted IL-10 and IL-12 cytokines under S protein stimulation. Dendritic cells would inhance the immune reaction of lymphocytes through these cytokines. We are now collecting the cord blood and blood from children to see if there is any different cytokines and chemokines secretion pattern.

About signaling pathway, we failed to transfect pcDNA to monocytes and dendritic cells. The transfection of A549 epithelial cell line demonstrated that the pcDNA-S and pcDNA-N or N protein may induce NF-κB activation. Chang et al found that S protein of SARS-CoV could induce release of IL-8 in the lung cells via activations of MAPKs and AP-1.[12] Further signaling pathyway should be studied.

In conclusion, the SARS-CoV E protein may induce proinflammatory cytokines and chemokines secretion of moncytes and S protein may induce the release of IL-10 and IL-12 in dendritic cells from healthy adults. Futher studies in young children and cord blood should be done to explore the mechanism of milder symptoms and less aggressive clinical course in children. The signaling pathway in monocytes dendritic cells should also be studied for the immunopathogenesis of SARS.

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延聘博士後研究成效評估	一、是否達到延聘之預期目標？ 二、研究的方法、專業知識及進度如何？ 三、受聘人之研究對該計畫(或貴單位)助益如何？ 四、受聘人於受聘期間對增進其研究能力及經驗之助益如何？ 五、具體工作績效或研究成果： 六、是否納編或移轉至其他單位或產業界？ 一．此一計畫的主要目的是要研究 SARS 病毒的蛋白對單核細胞、肺部上皮細胞和樹突細胞的影響。在這段期間，本人和指導研究助理分別完成了 N, E, M 及 S 蛋白對肺部上皮細胞的影響，同時也完成了對樹突細胞與單核細胞的細胞激素和趨化激素的影響。這些研究成果已經達到我們的預期目標。 二．本人在細胞免疫學上有著豐富的知識和技術，所以除了自己的研究進度外，也可以指導研究助理和研究生從事研究工作，對整個研究計畫扮演著關鍵的角色。 三．本人在擔任博士後研究這段期間，對整個研究計畫和敝研究室的研究進展扮演著一個舉足輕重的角色。 四．由於本人在博士研修期間主要是從事茶多酚對細胞訊息傳導的影響，而對病毒相關研究和細胞免疫學的研究較少接觸，所以經由參與此一研究計畫，可以讓她能夠對免疫學在病毒疾病的研究上有更多的涉獵。 五．目前已經完成 SARS 病毒的重組蛋白對肺部上皮細胞的活化，而且已經進一步利用純化出來的 N, E, M 和 S 蛋白與單核細胞和樹突細胞進行活化的研究，進一步分析這些細胞製造發炎性細胞激素和趨化激素的情形，這些研究結果也已經完成。而我們也進一步研究這些重組蛋白活化肺部上皮細胞的訊息傳導途徑，研究其活化的途徑，可以讓我們對 SARS 病毒的致病機轉有更清楚的了解。這些研究結果很適合在學術期刊發表。 六．個人還是希望博士後研究員在從事一段時間的研究工作後能夠繼續到學術界或是產業界繼續從事相關的研究工作。