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• *BRIEF REPORTS* •

Gene expression profiles in peripheral blood mononuclear cells of SARS patients

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Abstract

AIM: To investigate the role of inflammatory and anti-viral genes in the pathogenesis of SARS.

METHODS: cDNA microarrays were used to screen the gene expression profiles of peripheral blood mononuclear cells (PBMCs) in two SARS patients (one in the acute severe phase and the other in the convalescent phase) and a healthy donor. In addition, real-time qualitative PCR was also performed to verify the reproducibility of the microarray results. The data were further analyzed.

RESULTS: Many inflammatory and anti-viral genes were differentially expressed in SARS patients. Compared to the healthy control or the convalescent case, plenty of pro-inflammatory cytokines such as IL-1, TNF-α, IL-8, and MAPK signaling pathway were significantly upregulated in the acute severe case. However, anti-inflammatory agents such as IL-4 receptor, IL-13 receptor, IL-1Ra, and TNF-α-induced proteins 3 and 6 also increased dramatically in the acute severe case. On the contrary, a lot of IFN-stimulated genes like PKR, GBP-1 and 2, CXCL-10 and 11, and JAK/STAT signal pathway were downregulated in the acute severe case compared to the convalescent case.

CONCLUSION: Gene expression in SARS patients mirrors a host state of inflammation and anti-viral immunity at the transcription level, and understanding of gene expression profiles may make contribution to further studies of the SARS pathogenesis.

Key words: SARS pathogenesis; Gene expression profiles; cDNA microarray; Inflammation response; Innate anti-viral immunity

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INTRODUCTION

A new contagious disease occurred in 2003[1-3], which lasted for at least 6 mo and swept over 29 countries in the world, causing numerous deaths and triggering public panic[4]. However, it took less than 2 mo to successfully identify the causative agent-a novel coronavirus[3,5]. Meanwhile, investigation of the unique pathogenic mechanism of this disease is still challenging and intriguing. Clinical data suggest that it is an abnormal pathological reaction to pulmonary viral infection characterized by acute lung injury[1,2,4,6] that determines the process of the symptoms. Acute lung injury is a multi-factorial, pathophysiological process involving cytokines and adhesion molecules, as well as inflammatory and immune cells[6-8]. Many pro- and anti-inflammatory cytokines such as IL-1, TNF-α, IL-8, IL-4, and IL-10 have been demonstrated to play a pivotal role in the pathogenesis of acute lung injury and severe systemic inflammation[6,8-11]. To determine the role of cytokines in the pathogenesis of SARS, immunological techniques such as RIA, ELISPOT, and ELISA have been employed to measure cytokine alterations in blood samples from SARS patients[12-14]. Jones *et al*.[12], reported that the number of IFN-γ, IL-2, IL-4, IL-10, and IL-12 secreting cells induced by T-cell activators is below normal in many or most patients, while the number of cells which are induced to produce IL-6 and TNF-α by T-cell or monocyte activators is higher than normal in many early SARS patients, and increases in some SARS patients during and after treatment. Wong *et al*.[13], found that Th1 cytokine, inflammatory cytokines such as IL-1, IL-6, and IL-12 and chemokines such as IL-8, MCP-1, and IP-10 are increased. Furthermore, Zhang *et al*.[14], revealed that there is a difference in relationship between IL6, IL-8, TGF-β concentration, and SARS severity (positive for IL-6, but negative for IL-8 and TGF-β). Although these studies have shown the evidence of activated Th1 cell-mediated immunity and the hyper-innate inflammatory response, the role of these cytokines in the pathogenesis of the severe systemic inflammation and the mechanisms underlying the pathogenesis of SARS need to be further studied.

Development of microarray technology has provided a powerful tool for study of the complicated biological process in cells and tissues. cDNA microarray is used to analyze the virus-host cell interactions, and improvements have been achieved in the diagnosis, treatment, and prevention of infectious diseases[15]. Therefore, in this study, we used cDNA microarray to analyze the global gene expression profiles of peripheral blood mononuclear cells (PBMCs) from two SARS patients, one in the acute severe phase and the other in the convalescent phase. The results may make contribution to studies of the SARS pathogenesis.

MATERIALS AND METHODS

Patients

Shanghai Municipal Hospital for Infectious Diseases was the appointed hospital for SARS patients during the SARS outbreak in Shanghai area. A total of seven patients with SARS were accepted for treatment in this hospital from 2nd May to 20th August 2003. In this study, two SARS patients in different clinical courses were enrolled, one in the acute severe phase (1 wk after admission to hospital and died 1 d after blood samples were taken) and the other in the convalescent phase (about 1 mo after admission). After admission to the hospital, both patients received the standard treatment. Additional clinical information is summarized in Table 1.

Table 1 Clinical characteristics of two SARS patients

	P1	P2
Age (yr)	57	40
Gender	Male	Female
Body temperature (℃)	39.2	36.8
White cell count ($\times 10^9$/L)	13.30	4.67
Neutrophil ($\times 10^9$/L)	12.80	3.25
Lymphocyte ($\times 10^9$/L)	0.406	0.897
$CD3^+$ (/μL)	359	791
$CD4^+CD3^+/CD3^+$ (%)	86	58
$CD8^+CD3^+/CD3^+$ (%)	13	36
$CD4^+CD8^+CD3^+/CD3^+$ (%)	4	2
$CD4^+/CD8^+$	6.38	1.62
Monocyte ($\times 10^9$/L)	0.122	0.442
Eosinophil ($\times 10^9$/L)	0.004	0.033
Basophil ($\times 10^9$/L)	0.009	0.041
Outcome	Death	Rehabilitation

Blood samples and RNA isolation from peripheral blood mononuclear cells

Blood samples (5 mL each) were collected from two patients and a healthy donor with anticoagulant at bedside. PBMCs in lymphocyte separation medium (Sigma, USA) were isolated. Total RNA was isolated using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. After TRIzol purification, RNA was repurified by phenol-chloroform extraction and ethanol precipitation, and quantified by spectrophotometry. In addition, RNA samples were electrophoresed on 2.2 mol/L 0.7% agarose-formaldehyde gel and visualized by ethidium bromide staining to ensure that there was not overt RNA degradation.

Microarray hybridization and data analysis

Microarray hybridization was performed by Shanghai Genentech Company according to the standard Affymetrix protocol. In brief, RNA from two patients and a healthy control was converted to cDNA with Superscript™ II RT (Invitrogen, USA) and then to biotin-labeled cRNA with RNA transcript labeling kit (Affymetrix, USA). cRNA was cleaned up and qualified and then fragmented for hybridization. After hybridization to the human HG-U113A GeneChip containing approximately 13 000 unique genes or expression-signature tags (Affymetrix), the gene chips were automatically washed and stained with streptavidin-phycoerythrin by a fluidics system. After the chips were scanned with a GeneArray scanner (Hewlett-Packard, USA), gene transcript values were determined using algorithms in the Microarray Analysis Suite Software (5.0 version, Affymetrix). Each chip was scaled to an overall intensity of 1 500 to correct for minor differences in the overall chip hybridization intensity and to allow comparison between chips. Data were normalized to the average of the healthy control. The gene lists of two patients containing genes with $P<0.05$ were put out in the style of Excel files.

Real-time quantitative PCR

Real-time quantitative PCR of cDNA samples from two patients and a healthy control was carried out in triplicate with the indicated primers (Table 2), at a volume of 20 μL using FastStart DNA Master SYBR Green I Mixture Kit® (Roche Diagnostics, USA) in a LightCycle® system (Roche Diagnostics, USA). Initial denaturing for 10 min at 95 ℃ was followed by 45 cycles at 95 ℃ for 10 s, at 55 ℃ for 15 s, and at 72 ℃ for 20 s. Detection of the fluorescent products was set at the last step of each cycle. To determine the specificity of amplification, melting curve analysis was applied to all final PCR products, after the cycling protocol. In addition, template-free negative controls were run with each gene specific primer. PCR for RNA products of three samples was performed in order to exclude genomic DNA contamination. The standard curve was prepared with a serial of dilutions of genomic cDNA from a GAPDH-containing plasmid. Results were representative of three independent experiments.

Table 2 Primer sequence used in real-time quantitative PCR analysis

Accession number	Description	Forward primer (5′→3′)	Reverse primer (5′→3′)
NM_000634	IL-8R α	GGAACTGGTGTCTTCAGGG	CATCTAATGTCAGATTCGGGG
NM_003855	IL-18R1	GGGTATTACTCCTGCGTGCA	CCATTTTCTTCCCCGAACATCC
BC025925	GAPDH	GGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC

RESULTS

Validation of microarray results

To verify the reproducibility of the microarray results, 2 genes (IL-8 receptor-α and IL-18 receptor-1) were selected and tested by real-time quantitative PCR. GAPDH was used as internal control to normalize the total RNA. The ratios of the signal intensity of specific genes to GAPDH, as well as their comparison to microarray results, are listed in Table 3. As shown in Table 3, the results of RT-PCR analysis were aligned with those from the microarray analysis, suggesting the creditability of our microarray results.

Table 3 Comparison of microarray and real-time quantitative PCR analysis on selected genes (mean±SD)

Gene description	Techniques	P1	P2
IL-8R α	Microarray	73.52	–
	Real-time qPCR	11.63±0.15	0.37±0.09
IL-18R1	Microarray	42.33	–
	Real-time qPCR	48.55±0.36	0.38±0.27

Global characteristics of gene expression in PBMCs of SARS patients

We uploaded the genes altered over 2.0-fold (at http://www.mvlab-fudan.cn/part9.htm) to Affymetrix online data analysis system (https://www.affymetrix.com/analysis/netaffx/batch_query.affx), and then got an illustration of hierarchical structure according to their biological process. The number of genes changed over 2.0-fold in two SARS patients is summarized in Table 4. Results showed that many genes including those for cell communication, cellular physiological process, death, metabolism, organismal physiological process, and response to stimulus changed significantly in both SARS patients. The number of genes changed in the acute severe case, was much higher than that in the convalescent case.

Table 4 Functional categories of over 2.0-fold-regulated genes in two SARS patients

Classification	P1	P2
Cell communication	802 (518 ↑ 284 ↓)	288 (116 ↑ 172 ↓)
Cell differentiation	65 (33 ↑ 32 ↓)	24 (6 ↑ 12 ↓)
Cellular physiological process	1 056 (645 ↑ 411 ↓)	406 (188 ↑ 218 ↓)
Coagulation	42 (25 ↑ 17 ↓)	17 (6 ↑ 11 ↓)
Development	316 (202 ↑ 114 ↓)	132 (50 ↑ 82 ↓)
Death	166 (116 ↑ 50 ↓)	80 (45 ↑ 35 ↓)
Extracellular structure organization and biogenesis	3 (1 ↑ 2 ↓)	2 (2 ↓)
Homeostasis	30 (13 ↑ 17 ↓)	11 (8 ↑ 3 ↓)
Metabolism	1 497 (867 ↑ 630 ↓)	512 (191 ↑ 330 ↓)
Obsolete biological process	1 (1 ↓)	1 (1 ↓)
Organismal physiological process	467 (278 ↑ 189 ↓)	198 (110 ↑ 88 ↓)
Pathogenesis	1 (1 ↑)	2 (2 ↑)
Regulation of cell process	57 (57 ↓)	88 (48 ↑ 40 ↓)
Response to stimulus	532 (317 ↑ 215 ↓)	232 (124 ↑ 108 ↓)
Viral life cycle	15 (7 ↑ 8 ↓)	10 (5 ↑ 5 ↓)
Behavior	14 (8 ↑ 6 ↓)	6 (1 ↑ 5 ↓)
Total	3 854 (2 273 ↑ 1 581 ↓)	1 380 (527 ↑ 853 ↓)

Differentially expressed genes involved in inflammation and immune response in SARS patients

In the light of a pivotal role in the pathogenesis of SARS, the genes involved in inflammation, immune response or anti-viral effect are categorized in Tables 5-7.

As shown in Table 5, the pro- and anti-inflammatory cytokine genes were differentially expressed in two patients. Compared to healthy control, the genes encoding IL-1, IL-8, TNF-α, and ICAM-1 increased by 3.73, 8.00, 17.15 and 17.15 folds respectively in the acute severe case. Type I IL-1 receptor, TNFRSF1A, IL-8 receptor, IL-18 receptor 1 also increased by 18.38, 4.00, 73.52/51.98, and 42.22 folds respectively in the acute severe case. However, all of them did not change in the convalescent case. In addition, many anti-inflammatory agents were also remarkably upregulated in the acute severe case. Inhibitors of IL-1 and TNF-α signal pathway such as type II IL-1 receptor, soluble type II IL-1 receptor, IL-1Ra, IRAK3, soluble IL-1 receptor accessory protein, TNFRSF10C, TNF-α-induced proteins 3 and 6 were upregulated by more than 10-folds in the acute severe case, but did not change or were expressed at low level in the convalescent case.

As shown in Table 6, in 21 IFN-related molecules, most of the IFN-related molecules except for IFNGRs and IFNAR1 were not upregulated in the severe case, but they were upregulated more than two-fold in the convalescent case. For the 12 chemokine-related genes detected in this microarray, RANTES (a marker of activated T cells) and CX3CR1 were strikingly downregulated in the acute severe case. Two viral RNA-recognized TLRs (TLR-3 and -7), especially TLR-7, were significantly downregulated in the acute severe case. The above results indicated that there was dysfunction of the innate immune responses in the acute severe case.

The altered genes which could play a vital role in the process of stress, inflammation, and immune response are summarized in Table 7. In comparison to the convalescent case, the JAK/STATs were suppressed in the acute severe case. The expression of STATs 1, 2, and 4 were not upregulated, while MAPKs were upregulated in the acute severe case as compared to the healthy control. Six out of seven components of MAPK signal cascade were upregulated.

DISCUSSION

As an emerging disease, attention has been paid to the high infectivity and virulence of SARS. In the course of the disease, the important observation is lymphopenia and the depletion of T-lymphocyte subsets in most SARS cases, indicating the immunity dysfunction in this readily-transmissible disease, particularly during its early phase[7]. Another characteristic of the disease is acute lung injury accompanied with signs of the systemic inflammation, the duration and intensity of which are closely associated with the severity and prognosis of the disease[1,2,4].

A typical feature of all inflammatory disorders is the excessive recruitment of leukocytes to the inflammation site, which is a well-orchestrated process involving several protein families, including pro-inflammatory cytokines, chemotactic cytokines, and adhesion molecules[9,10]. This

Table 5 Differentially expressed genes encoding pro- and anti-inflammatory cytokines are involved in IL-1 and TNF-α signaling cassettes in two patients

GenBank access	Definition	P1	P2
Pro- and anti-inflammatory cytokines and receptors			
AF043337	IL-8	8.00	–2.46
NM_000634	IL-8 receptor, α	73.52	–
NM_001557	IL-8 receptor, β	51.98	4.29
NM_000575	IL-1, α	3.73	–
NM_000576	IL-1, β	6.50	11.31
NM_000877	IL-1 receptor, type I	18.38	–
NM_004633	IL-1 receptor, type II	362.04	3.84
U64094	Soluble type II IL-1 receptor	630.35	–
NM_003856	IL-1 receptor-like 1	11.31	3.25
U65590	IL-1 receptor antagonist IL-1Ra	14.93	–
AF051151	Toll/IL-1 receptor-like protein 3	14.93	–
NM_000418	IL-4 receptor	13.93	–
NM_000600	IL-6	–	3.03
NM_002184	IL-6 signal transducer gp130	–	–2.14
BC001903	IL-10 receptor, β	4.00	–
NM_004512	IL-11 receptor, α	–6.06	–2.30
NM_001560	IL-13 receptor, α1	3.25	–
U62858	IL-13 receptor	5.66	–
U81380.2	IL-13 receptor soluble form	4.29	–
NM_000585	IL-15	–3.03	2.00
NM_004513	IL-16	–	–2.83
NM_014339	IL-17 receptor	4.29	–
NM_003855	IL-18 receptor 1	42.22	–
NM_003853	IL-18 receptor accessory protein	19.70	–
AF269133	Novel interleukin receptor	–3.03	–
NM_004862	TNF-α	17.15	–
NM_001065	TNFRSF1A	4.00	–
NM_003841	TNFRSF10C	19.70	–
NM_000760	G-CSF 3 receptor	25.99	–
BC002635	GM-CSF 2 receptor, α, low-affinity	3.03	–
M64445	GM-CSF receptor	4.92	2.64
NM_002607	Platelet-derived growth factor α polypeptide	–2.46	–
NM_004347	Caspase 5	4.59	–
NM_000201	Intercellular adhesion molecule 1	17.15	–
NM_002162	Intercellular adhesion molecule 3	6.28	–
NM_003243	Transforming growth factor, β receptor III	–36.76	–4.59
NM_003242	Transforming growth factor, β receptor II	–	–2.30
NM_000358	Transforming growth factor, β-induced, 68 ku	–24.25	–
The genes involved in the IL-1 signaling pathway			
NM_007199	IL-1 receptor-associated kinase M	19.70	–
NM_002182	IL-1 receptor accessory protein	25.99	–
AF167343	Soluble IL-1 receptor accessory protein	55.72	–
M87507	IL-1 β convertase	3.25	–
U13698	IL-1-β converting enzyme isoform γ	3.03	–
U13699	IL-1-β converting enzyme isoform δ	–	2.14
U13700	IL-1-β converting enzyme isoform ε	2.64	–
The TNF signal downstream genes			
NM_004619	TNF receptor-associated factor 5	–	–2.00
NM_016614	TRAF and TNF receptor-associated protein	3.84	–2.30
NM_006290	TNF-α induced protein 3	5.66	–
NM_007115	TNF-α induced protein 6	45.25	4.92
AB034747	Small integral membrane protein of lysosome late endosome	7.46	–
U50062	RIP protein kinase	–	–2.00

process is resolved by anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF-β[10]. The well-known pro-inflammatory cytokines include IL-1 and TNF-α, which are induced as the signals by pattern recognition receptors (like TLRs) and initiate activation of a series of signal transduction networks to release mediators, prompting inflammation and immunity[10,16,17]. In our study, pro-inflammatory cytokines (like IL-1, IL-8, IL-17, IL-18, TNF-α

Table 6 Differentially expressed genes involved in immune regulation in two SARS cases

GenBank access	Definition	P1	P2
IFN and IFN-induced genes			
M29383	IFN-γ	–	2.14
NM_000416	IFN-γ receptor 1	4.59	–
NM_005534	IFN-γ receptor 2	3.84	–
NM_000629	IFN (α, β, and ω) receptor 1	4.29	–
NM_002198	IFN regulatory factor 1	–	3.84
NM_002460	IFN regulatory factor 4	–	–2.30
NM_004030	IFN regulatory factor 7	–	3.25
BC001356	IFN-induced protein 35	–	2.83
M34455	IFN-γ-inducible indoleamine 2,3-dioxygenase	–	5.66
NM_001548	IFN-induced protein with tetratricopeptide repeats 1	–	3.03
NM_001549	IFN-induced protein with tetratricopeptide repeats 4	–	6.06
NM_002053	Guanylate binding protein 1, IFN-inducible	–	3.03
NM_004120.2	Guanylate binding protein 2, IFN-inducible	–	3.73
NM_022873	IFN-α-inducible protein	–	6.50
NM_003641	IFN-induced transmembrane protein 1 (9-27)	–	2.30
NM_004509	IFN-induced protein 41	–	2.14
NM_005532	IFN-α inducible protein 27	–	14.93
NM_005101	IFN-stimulated protein, 15 ku	–	6.50
NM_006417	IFN-induced, hepatitis C-associated microtubular aggregate protein	–2.46	2.64
NM_002759	PKR	–	2.14
NM_002462	Mx1	–	2.14
Chemokines and receptors			
NM_001511	GRO1	55.72	–
NM_002993	Granulocyte chemotactic protein 2	3.73	–
NM_001565	CXCL10	–	14.93
AF030514	CXCL11	–	18.38
AJ224869	CXCR4	6.50	2.83
M21121	RANTES	–13.93	–
NM_001295	CCR1	3.84	–
NM_000648	CCR2	–9.19	–3.25
NM_001837	CCR3	–	6.50
NM_000579	CCR5	–3.25	–4.00
NM_001838	CCR7	–3.73	–2.64
U20350	CX_3CR1	–42.22	–
Toll-like receptors			
NM_003264	Toll-like receptor 2	8.00	–
NM_003265	Toll-like receptor 3	–3.25	–2.30
NM_003266	Toll-like receptor 4	3.73	–2.64
NM_016562	Toll-like receptor 7	–17.15	2.30

Table 7 Differentially expressed genes involving MAPK or JAK-STAT signaling pathways in two SARS cases

GenBank access	Definition	P1	P2
U35002	JNK2 β1 protein kinase	–2.00	–2.14
U31601	JAK-3B	10.56	–
NM_007315	STAT-1	–	2.14
NM_005419	STAT-2	–	3.03
NM_003151	STAT-4	–2.30	–
NM_012448	STAT5B	8.57	–
AB005043	STAT induced STAT inhibitor 1	5.66	3.03
NM_003955	STAT induced STAT inhibitor 3	4.92	–
NM_002745	MAPK 1	4.00	–
NM_002748	MAPK 6	3.73	–
NM_001315	MAPK 14	9.19	–
NM_004759	MAPK-activated protein kinase 2	4.29	–
NM_003668	MAPK-activated protein kinase 5	–2.46	–
NM_001674	Activating transcription factor 3	2.46	5.27
NM_007348	Activating transcription factor 6	3.73	–

and etc.) were highly expressed in the acute severe case and lowly expressed in the convalescent case. Real-time quantitative PCR of empirically selected genes also showed that pro-inflammatory cytokines were highly expressed. These findings, as expected, are consistent with the clinical stage[2,6,8], though individual variation and sensitivity of examination exist. In addition, anti-inflammatory agents (IL-4, 10, and 13 receptors) and agonists of IL-1 and TNF (type II IL-1 receptor, soluble IL-1 receptor, IL-1Ra, and TNF-α decoy receptor) increased dramatically in the acute severe case, which could constitute a negative feedback to robust inflammation or manifestations of systemic inflammation[16,17]. But whether the alteration is associated with virus replication or interaction between the viral and cellular proteins is to be further elucidated.

Chemokines are also important cytokines involved in inflammation, dendritic cell maturation, neutrophil degranulation,

antibody class switching and T-cell activation[18-20]. Furthermore, recent *in vitro* and *in vivo* findings support some members of chemokine system like CXCL9, CXCL10, and CXCL11 contribute to the resolution of viral infections[19,20]. Unfortunately, transcripts of IFN-induced chemokines[18,19] (RANTES, CXCL10, and CXCL11) were downregulated in the acute severe case, while inflammatory chemokines such as GRO-1, G-CSF3R, IL-8 and its receptors increased significantly. The expression profiles of chemokines as well as neutrophil predominance in white cells reflect rampant inflammation in the acute severe case[8,9].

Although the acute severe case is treated with IFN-α, poor expression of IFN-stimulated genes[21], TLR3, 7 and immune cell activation markers (CD antigens and MHC molecules, data not shown) may mirror the defect of host anti-viral immunity. Furthermore, some studies indicate that the activation of some pro-inflammatory cytokines such as IL-1, IL-6 or IL-8 signaling pathways interferes with the IFN signaling[22-25], let alone the action of IL-4, IL-10 or IL-13[9]. Outbreak of pro- and anti-inflammatory agents might interfere with the IFN signaling pathway, but direct interaction between coronavirus and IFN system cannot be excluded.

It is intriguing to find that, although the important genes involved in NF-κB signaling pathway were not detected in our microarrays, JAK/STAT signaling pathway was suppressed, another important signaling pathway associated with production of inflammatory cytokines, the MAPK signaling pathway[20,26,27], was upregulated in the acute severe case compared to the healthy control or convalescent case. It was reported that there are expression alterations in the MAPK pathway in different leukocytes from patients with SARS and virus infection and viral proteins exert effects on the MAPK signaling pathway in cell culture models[27-29]. But the relationship between changes of three signaling pathways and the SARS process needs to be further studied.

In conclusion, our results may partially reveal the different expression patterns of inflammatory and immune genes and related signal pathways at different phases of SARS pathological process. The overexpressed pro- and anti-inflammatory cytokines may contribute to acute lung injury and imbalance of homeostasis especially in acute severe phase. Thus our results may hopefully make a contribution to further studies of the SARS pathogenesis.

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