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Cytokine Regulation in SARS Coronavirus Infection Compared to Other Respiratory Virus Infections

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The pathogenesis of severe acute respiratory syndrome (SARS) is poorly understood and cytokine dysregulation has been suggested as one relevant mechanism to be explored. We compared the cytokine profile in Caco2 cells after infection of SARS coronavirus (SARS-CoV) with other respiratory viruses including respiratory syncytial virus (RSV), influenza A virus (FluAV), and human parainfluenza virus type 2 (hPIV2). Interferon (IFN) system (production and response) was not suppressed by SARS-CoV infection. Therefore, SARS-CoV replication was suppressed by pretreatment with IFN. SARS-CoV and RSV induced high levels of IL-6 and RANTES compared with FluAV and hPIV2. Induction level of suppressor of cytokine signaling-3 (SOCS3) by SARS-CoV was significantly lower than that by RSV in spite of the significant production of IL-6. Toll-like receptors 4 and 9, which correlate with the induction of inflammatory response, were upregulated by SARS-CoV infection. Collectively, overinduction of inflammatory cytokine and dysregulation of cytokine signaling may contribute to the immunopathology associated with ''severe'' inflammation in SARS. ***J. Med. Virol. 78:417–424, 2006.***

KEY WORDS: SARS-CoV; SOCS3; cytokine; IL-6; IFN; TLR

INTRODUCTION

Severe acute respiratory syndrome (SARS) is caused by a novel coronavirus, now called the SARS coronavirus (SARS-CoV). The main clinical feature is respiratory systems involvement, and additional gastrointestinal symptoms are also common [Leung et al., 2003; Nicholls et al., 2003]. Severely affected patients develop acute respiratory distress syndrome, which corresponds with diffuse alveolar damage. This acute lung injury is a complex and multifunctional pathophysiological process involving inflammatory cytokines released from stimulated macrophages in the alveoli lead to dysregulation of the immune system [Nicholls et al., 2003]. Cytokine profiles in sera of SARS patients showed that interleukin (IL)-6 and interferon (IFN)-γ, which promote inflammation by inducing cell injury, are significantly increased and the induction levels are closely correlated with the severity of SARS [Zhang et al., 2004; Huang et al., 2005]. It is well known that IL-6 induces the expression of suppressor of cytokine signaling-3 (SOCS3), a negative regulator of IL-6 signaling, through gp130-mediated STAT3 phosphorylation/activation [Yasukawa et al., 2003]. On the other hand, IL-10, which leads to a reduction in lung tissue injury, was increased in convalescent SARS patients [Zhang et al., 2004]. These results indicated that severe immune response, rather than virus virulence, contributes to the progressive damage to the lung in SARS and that some cytokines may play an important role in the onset and pathogenesis of SARS [Peiris et al., 2003]. However, the regulation system of cytokine production and signaling during SARS-CoV infection is poorly understood.

Infection of viruses to their susceptible host cells brings about induction of various cytokine types including IFN and ILs, which are involved in the antiviral defense front, and immune regulation. The IFN system is a powerful defense mechanism against virus infection. The system is divided into two processes, namely the IFN production process first, and subsequently the

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establishment of an antiviral state, which is due to IFN-inducible proteins such as 2′, 5′-oligoadenylate synthetase (2-5AS), ds-RNA-acitivated protein kinase (PKR), IFN-stimulated gene 20 (ISG20), and Myxovirus resistance protein A (MxA) [Samuel, 1991; Sen and Ransohoff, 1993; Fujii, 1994; Gongora et al., 1997; Goodbourn et al., 2000], through activation of IFN-JAK/STAT signaling pathway. The IFN production process consists of two steps, the former involves IFN-β production through activation of IFN regulatory factor 3 (IRF3) by the virus at an early stage infection and the latter is production of a large amount of IFN-α by primarily induced IFN-β through the formation of transcriptional heterodimer with IRF7 and IRF3 [Sato et al., 2000].

Many viruses are able to suppress the IFN system at various points. Suppression of IFN-β production or IFN-JAK/STAT signaling pathway results in reduction of IFN-α induction and antiviral function. It has been reported that influenza A virus (FluAV) and human parainfluenza virus type 2 (hPIV2) reduce IFN-β induction by suppression of IRF3 phosphorylation [Talon et al., 2000; Poole et al., 2002]. Furthermore, hPIV2 and respiratory syncytial virus (RSV) counteracts IFN signaling by degradation of STAT2 [Andrejeva et al., 2002; Ramaswamy et al., 2004]. Therefore, the effect of IFN on the replication of these viruses is diminished. In contrast, IFN is a favored candidate as a therapeutic agent of SARS, exhibiting anti-SARS-CoV activity in in vitro experiments and in in vivo treatment trials [Zhao et al., 2003; Cinatl et al., 2004; Sainz et al., 2004; Morgenstern et al., 2005]. From lines of evidence, SARS-CoV seems to be sensitive to IFN. It is important to clarify the influence of SARS-CoV upon the IFN system, namely suppression of IFN production and counteraction of IFN signaling by SARS-CoV infection, because many viruses have evolved a variety of strategies to counteract the antiviral effect of IFN.

The aim of this study is to analyze the innate immune response induced by SARS-CoV infection. In particular, induction of IL-6 is thought to play a key role in patients with SARS. Analysis of regulation system of both production and signaling pathway of IL-6 is also essential to understand the pathogenesis of SARS. Indeed, investigation of SOCS3 induction level is crucial because this factor acts as a negative regulator in IL-6 signaling. To characterize the cytokine regulation system in SARS, we examined the influence of SARS-CoV upon both IFN system and cytokine production in its infected cells as compared with in cells infected with RSV, FluAV, and hPIV2.

MATERIALS AND METHODS

Cells, Virus Infection, and IFN Treatment

The human colon carcinoma cell line Caco2, the bronchiolar carcinoma cell line A549 and the cervical squamous carcinoma cell line SiHa were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in minimum essential medium (Gibco-Invitorogen, Carlsbad, CA) containing 10% (V/V) heat-inactivated fetal bovine serum (Gibco-Invitorogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. SARS-CoV strain Hanoi was provided by Dr. Morita (Nagasaki University, Japan) [Hong et al., 2004]. Concentrated virus was then exposed to UV light in order to inactivate the virus. RSV strain Long was described previously [Tsutsumi et al., 1989]. FluAV is a clinical isolate (type AH3) from a patient with influenza in Hokkaido, Japan. Human PIV2 was provided by Dr. Ito (Mie University School of Medicine, Mie, Japan). All virus infections were done at a m.o.i. of 1.0. Virus titer (the 50% tissue culture infectivity does-TCID50/ml-) of SARS-CoV was determined using Vero E6 cells as described previously [Kariwa et al., 2004]. Human IFN-α and IFN-β were purchased from Serotec (Oxford, UK) and Genzyme-Techne (Minneapolis, MN), respectively. Both were used at a final concentration of 1,000 IU/ml. These experiments were performed in more than three times.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For RNA analysis, total cellular RNA was prepared from cell lines by using ISOGEN according the manufacturer's protocol (Nippon Gene, Toyama, Japan). RT-PCR assay was performed using One-step RT-PCR kit (QIAGEN, Hilden, Germany). The quantitative nature of the PCR was validated by the linearity of the determination curve at various concentrations of RNA. The sequences of the primers (Sigma-Genosys, Ishikari, Japan) are given in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a control. These experiments were performed in triplicate.

RESULTS

Virus Infection

Three cell lines, Caco2, A549, and SiHa, were investigated for their permissiveness to SARS-CoV infection. Angiotensin converting enzyme 2 (ACE2) has been identified as a receptor of SARS-CoV in different cell types [Li et al., 2003]. Indeed, ACE2 is expressed at a high level in the primary target cell of SARS-CoV [To and Lo, 2004]. Therefore, we examined the expression level of the ACE2 mRNA in these cell lines by RT-PCR and found a markedly higher expression level of the mRNA in Caco2 than A549 and SiHa cells (Fig. 1). Furthermore, a cytopathic effect (CPE) caused by SARS-CoV developed in Caco2 cells at 24 hr post infection. A large amount of the infected cells were died on third day post infection. A549 and SiHa cells did not show any CPE for 4 days after infection. Virus mRNA of SARS-CoV M protein was found in Caco2 cells infected with SARS-CoV by RT-PCR, but not in infection of A549 and SiHa cells.

IFN Production

It was reported that many viruses including RSV, FluAV, and hPIV2 have evolved several anti-IFN

TABLE I. Oligonucleotide Primers Used for Semi-quantitive Reverse Transcription-polymerase Chain Reaction

Target gene	Primer sequence; forward; 5′-3/reverse; 5′-3′
SARS/M	tggcagacaacggtactatt/taaggtaccttactgt
SARS/N	tgtctgataaatggacccc/gtcatcatcagtgtttatgc
SARS/E	tgtactcattcgtttcggaag/ttagaccagaagatcaggaac
ACE2	cattggagcaagtgttggatctt/gagctaatgcatgccattctca
IFN-β	gtctcctccaaattgctctc/acaggagcttctgacactga
IFN-α1	tggctggtaagaaatacttccg/tgttttcatgttggaccagatg
IFN-α2	cctgatgaaggaggactccatt/aaaaaggtgagctggcatacg
IFN-λ1	atgactggggactgcacgccagtg/tcagacacacaggtccc-cactggcaacaca
IFN-λ2	atggctgcagcttggaccgtgg/tcaggtggactcagggtgggttga
IRF7	gcccttacctcccctgttat/ccactgcagcccctcatag
2-5AS	ccaggaaattaggagacagc/tggcagggaggaagcaggac
ISG20	ccagaattctggctgggagccgtgaggtg/tgaggtacctcagtctga-cacagccaggc
M × A	gcatcccaccctctattact/tgtcttcagttcctttgtcc
PKR	ttggctcaggtggatttgg/ggcttttcttccacascagtc
IRF-1	ccagagaaaagaaagaaagtcg/cacatggcgacagtgctgg
IL-6	atgaactccttctccacaagcgc/gaagagccctcaggctggactg
IL-8	acttagatgtcagtgcataaagac/ttatgaattctcagccctcttcaa
RANTES	acaggtcaaactacaactcca/tcagctcttagcagacattgg
TLR4	agatggggcatatcagagc/ccagaaccaaacgatggac
TLR7	agtgtctaaagaacctgg/cttggccttacagaaatg
TLR9	ttatggacttcctgctggaggtgc/ctgcgttttgtcgaagacca
SOCS1	ccactccgattaccggcgcatc/gcttcctgcagcggccgcacg
SOCS3	tcacccacagcaagtttcccgc/gttgacggtcttccgacagagatgc
CIS	cagtgcaggaggccacatag/ggaggatctgctgtgcatag
GAPDH	tccaccaccctgttgctgta/accacagtccatgccatcac

function [Talon et al., 2000; Andrejeva et al., 2002; Poole et al., 2002; Ramaswamy et al., 2004]. However, it is still unclear whether SARS-CoV is able to induce several types of IFN. To investigate IFN-inducibility by SARS-CoV infection, we compared expression levels of several IFNs (IFN-α1, IFN-α2, IFN-β, IFN-λ1, IFN-λ2) mRNA in SARS-CoV infected cells with those in other viruses, RSV, FluAV, and hPIV2 infected cells. Viral infected cells were harvested to isolate cellular RNA after appearance of CPE caused by infected virus. As shown in Figure 2, all types of IFNs were little induced by hPIV2. Furthermore, little (IFN-λ1 and IFN-λ2) or lower levels (IFN-α and IFN-β) of IFNs induction was found in FluAV infected Caco2 cells. Indeed, in bioassay system, we cannot detect IFN activity in culture medium from FluAV and hPIV2 infected cells. In contrast, significant levels of IFN-α, IFN-β, and IFN-λ mRNA were detectable in SARS-CoV and RSV infected cells, and 2–32 IU/ml of IFN activity was recognized in culture medium of these viruses infected cells. The expression level of IFN-β mRNA by SARS-CoV was always higher than that in RSV infection. Induction of IFN-β and subsequent expression of IRF7 is an essential process to produce IFN-α. We found that IRF7 expression level in SARS-CoV and RSV infected cells was significantly higher than in other respiratory virus infections. These differences in IRF7 expression levels among viruses seemed to correlate with the level of IFNs production, including IFN-λs. However, a small amount of IFN-αs was induced in FluAV infection, although no IRF7 was induced. The IFN-α induction may be due to activation of IRF5, which contributes to IFN-α induction independently from IRF7 pathway [Island et al., 2002]. Furthermore, there was a correlation between production of IFNs and expression of IFN-stimulated genes (ISGs), 2-5AS, ISG20, and MxA. Expression of ISGs in SARS-CoV and RSV infected cells was higher than it was in FluAV and hPIV2 infected cells. The low level of ISGs induction in FluAV may be caused by a low titer (no detection in bioassay system) of secreted IFN in culture medium.

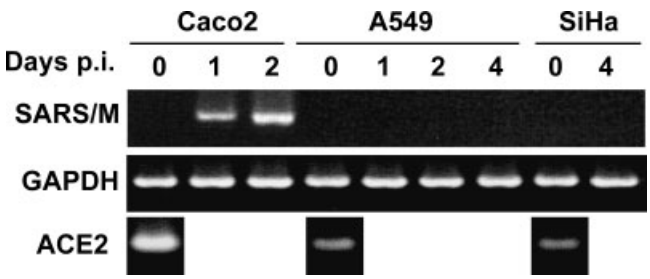

Fig. 1. Expression of severe acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin converting enzyme 2 (ACE2), and permissiveness of human colon epithelial cell line Caco2, human bronchiolar epithelial cell line A549 and human cervical squamous epithelial cell line SiHa to SARS-CoV infection. Expression of ACE2 mRNA was determined by semi-quantitive reverse transcription-polymerase chain reaction (RT-PCR). Permissiveness of cells to SARS-CoV was determined by detection of mRNA of SARS-CoV membrane protein (SARS/M) by semi-quantitive RT-PCR. Viruses were infected at m.o.i. 1.0, and the cells were harvested at various times indicated in the figure. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a control. p.i.; post infection.

IFN Signal Transduction and Anti-Viral Activity

Several viral proteins are known to suppress the IFN signal transduction pathway [Goodbourn et al., 2000]. To assess whether SARS-CoV infection interferes with IFN signaling, we investigated mRNA expression levels of ISGs, such as IRF1, 2-5AS, PKR, MxA and ISG20, in virus infected cells. The mRNA levels of ISGs in uninfected Caco2 increased by treatment with IFN-α or IFN-β for 4 hr (Fig. 3, control). After infection of the cells with SARS-CoV for 2 days, the infected cells were treated with IFN-α or IFN-β for 4 hr. As shown in Figure 3, mRNA induction levels of ISGs in the infected cells were similar to those in uninfected cells treated with IFNs. In contrast, these ISGs 'mRNAs were not induced by IFN treatment in hPIV2 infected cells (data not shown) due to suppression of IFN signaling pathway. These results show that SARS-CoV did not have counteracting strategies against establishment of IFN-induced antiviral state (IFN signaling). It is postulated that SARS-CoV replication is efficiently subdued by IFN because of the fact that it has few counteracting activities to IFNs.

Before virus infection, Caco2 cells were treated with IFN-α or IFN-β for 24 hr to examine the anti-viral effect of IFNs on the virus infection. Transcription of virus genes (E, M, and N) in the virus-infected cells was

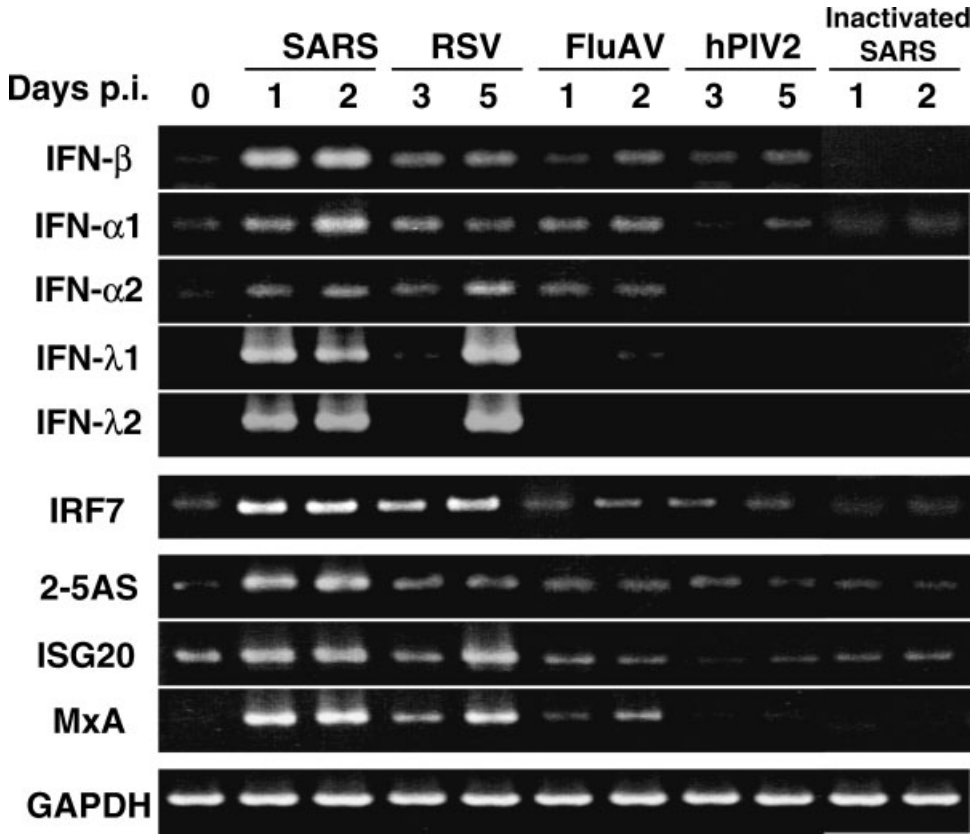

Fig. 2. Semi-quantitative RT-PCR analysis of mRNAs for interferons (IFNs), IFN regulatory factor (IRF) 7 and antiviral protein genes, 2′, 5′-oligoadenylate synthetase (2-5AS), IFN-stimulated gene 20 (ISG20) and myxovirus resistance protein A (MxA) in Caco2 cells during infection with SARS-CoV, respiratory syncytial virus (RSV), influenza A virus (FluAV), and human parainfluenza virus type 2 (hPIV2). Viruses were infected at m.o.i. 1.0. Cytopathic effects were observed on day 1 (SARS-CoV), day 3 (RSV), day 1 (FluAV), and day3 (hPIV2). GAPDH mRNA was determined as a control.

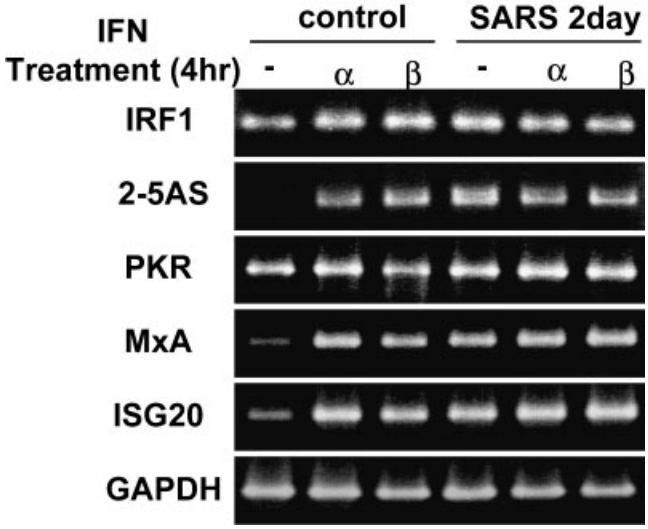

Fig. 3. Influence of SARS-CoV infection on induction of IFN-inducible antiviral protein mRNA. Caco2 cell were infected with SARS-CoV at m.o.i. 1.0. After 48 hr of infection, SARS-CoV infected Caco2 cells and uninfected cells were treated with 1,000 IU/ml IFN-α or IFN-β for 4 hr. The mRNAs of IRF1, 2–5AS, double-strand RNA dependent protein kinase (PKR), MxA and ISG20 were determined by semi-quantitive RT-PCR. GAPDH mRNA was determined as a control.

significantly reduced on the second day p.i. (Fig. 4). IFN-β in particular disclosed a more potent anti-viral activity than IFN-α. These results were also confirmed by reduction of infectious progeny virus production (Table II).

Inflammatory Response During Viral Infection

The initial step in immune response to a viral infection is the induction of proinflammatory cytokines and chemokines. They are strictly regulated by each other in the defense system or innate immunity. However, recent studies showed that overexerted immunereresponse in SARS-CoV infection may contribute to the progressive damage of the lung [Peiris et al., 2003]. To clarify the characteristics of cytokine induction profile in cells infected with SARS-CoV, we compared the mRNA induction pattern of cytokines in SARS-CoV with those in other respiratory viruses. Expression of IL-8 increased in all the viral infections examined, and the levels were almost the same among these viruses (about 10–35 ng/ml of protein level), with exception of FluAV (Fig. 5). Another chemokine, regulated upon activation normally T-cell expressed and secreted (RANTES) was also induced during SARS-CoV and RSV infections

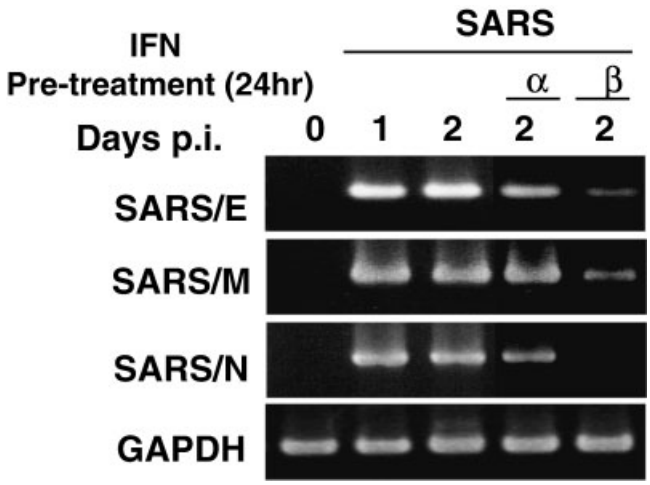

Fig. 4. Effect of IFN treatment on replication of SARS-CoV in Caco2 cells. After treatment with 1,000 IU/ml IFN-α or IFN-β for 24 hr, cells were infected with SARS-CoV at m.o.i. 1.0 for 48 hr. The cells were harvested on day 1 and day 2 after infection. The mRNAs of SARS-CoV were detected by semi-quantitive RT-PCR. E; envelope protein gene, M, membrane protein gene; N, nucleoprotein gene. GAPDH mRNA was determined as a control.

(about 1.0–10 ng/ml of protein level), whereas it was not detectable in FluAV and hPIV2 infections. Similarly to RANTES, proinflammatory cytokine IL-6 was dramatically increased to identical levels in both SARS-CoV and RSV infections (about 1.0–2.0 ng/ml of protein level), but not in FluAV and hPIV2 infections. In contrast, UV-inactivated SARS-CoV (Fig. 5) and RSV (data not shown) were not able to induce IL-6 mRNA expression. The cytokine induction profile is consistent with the results obtained in sera of patients with SARS [Zhang et al., 2004].

In general, cytokine signaling pathway is correctly regulated by negative feedback factors such as SOCS family [Kubo et al., 2003]. These factors are also induced in response to stimulation by appropriate cytokines, including IL-6 or IFNs via JAK/STAT signaling pathway. In the case of IL-6, Jak-1, Jak-2, and Tyk-2 are tyrosine kinases recruited to the gp130 receptor of IL-6, and these in turn activate the STAT3 to induce feedback inhibitor SOCS3, which acts as a negative regulator of IL-6 signaling [Kubo et al., 2003]. SOCS3 is, therefore, important for normal IL-6 signal transduction pathway. Less induction of SOCS3 results in continuous activation of STAT3, and leads to enhanced and prolonged IL-6 signaling. It is crucial to investigate the induction levels of SOCS3 in SARS-CoV and RSV infected cells. The expression of SOCS3 mRNA was detected in both of these virus infected cells. The level of SOCS3 in RSV infection was remarkably higher than in SARS-CoV infection (Fig. 5). No expression of SOCS3 was found in FluAV and hPIV2 infections, which produced no IL-6. Another specific negative regulator for STAT5 signaling pathway, cytokine-inducible SH2 protein (CIS), was also found in only RSV infection (Fig. 5) [Yoshimura, 1998]. These results indicate that dysfunction of the negative feedback system of cytokine signaling bring about strong induction of inflammatory reaction.

TABLE II. Reduction of Progeny Virus Release After IFN Treatment

IFN treatment	Virus titer (TCID 50/ml)
None	6.7×10^7
IFN-α	1.3×10^6
IFN-β	5.5×10^5

Caco2 cells were pretreated with 1,000 IU/ml of IFN-α or IFN-β for 24 hr, and then the cells were washed and infected with SARS-CoV. After cultivation for 2 days, the culture mediums were harvested to determined virus titer. Virus yields of SARS-CoV were calculated as a 50% tissue culture infectious dose.

Toll-like receptors (TLRs) play crucial roles in the pattern recognition of various microbial components [Takeda and Akira, 2001]. TLRs trigger a host defense response via the gene expression of inflammatory cytokines, as innate immune responses. It has been reported that TLR4 and TLR9 are essential for recognition of gram-negative bacterial lipopolysaccharide (LPS) and unmethylated CpG DNA, respectively [Poltorak et al., 1998; Hemmi et al., 2000; Lund et al., 2004]. The mRNA expressions of these TLRs were upregulated to a similar extent in SARS-CoV, RSV, and FluAV infections, but weak induction was found in hPIV2 infection (Fig. 5). UV inactivated SARS-CoV was never influence the cytokine regulation.

DISCUSSION

In this study, we investigated the influence of SARS-CoV on IFN system and proinflammatory cytokine production to clarify the pathogenesis of this virus and its susceptibility to IFN. The front line of defense against virus infection requires IFN-β production in virus infected cells, followed by establishment of antiviral functions through activation of IFN-JAK/STAT signal transduction pathway [Stark et al., 1998]. This IFN system is also known as an innate immune system.

Our results showed that RSV, FluAV, and hPIV2 produce less IFN-β than SARS-CoV infection. The multifunctional NS1 protein of FluAV and V protein of hPIV2 inhibit activation of IRF3, followed by suppression of IFN-β production [Talon et al., 2000; Poole et al., 2002]. Human PIV2 counteracts IFN signaling by degradation of STAT2 [Andrejeva et al., 2002]. Because of the limited production of IFN-β and dysfunction of JAK/STAT signaling pathway, the subsequent expression of ISGs were undetectable in hPIV2 (Fig. 2). In contrast, the results from Figures 2 and 3 show that IFN system (IFN production and IFN signaling pathway) is not suppressed at all in SARS-CoV infection. This is also confirmed by treatment of SARS-CoV infected cells with exogenous IFN (Fig. 3). However, it has been reported that there was little or no induction of beta IFN in SARS-CoV infected macrophages [Cheung et al., 2005]. Recently, Spiegel et al. [2005] , noted that SARS-CoV appears to block a step after the early nuclear transport of IRF3 in human 293 cells. These results for IFN-β

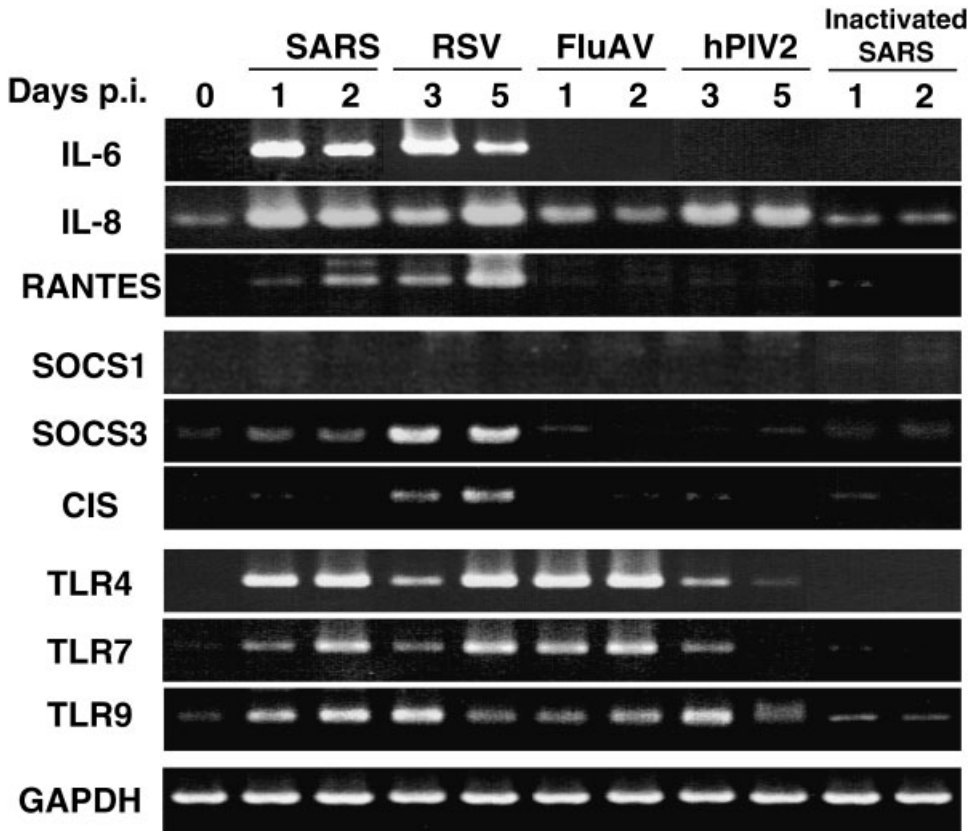

Fig. 5. The mRNAs of interleukin (IL)-6, IL-8, regulated upon activation normally T-cell expressed and secreted (RANTES), suppressor of cytokine signaling (SOCS)1, SOCS3, cytokine-inducible SH2 protein (CIS), and Toll like receptors (TLR)4, TLR7, and TLR9 during respiratory virus infection in Caco2 cells. Experimental condition of virus infection was the same as in Figure 2. Expression levels of mRNA were determined by semi-quantitive RT-PCR. GAPDH mRNA was determined as a control.

production differ from our data. The reason of this discrepancy is still unclear. It may be caused by different type of cells. Because of little contribution of SARS-CoV on IFN signaling, IFN is considered to be a suitable candidate for treatment of SARS-CoV infection. Though the efficiency of IFN treatment of SARS patients cannot be ascertained [Zhao et al., 2003; Fujii et al., 2004], some in vitro experiments showed significant affect of IFN against SARS-CoV replication [Cinatl et al., 2004; Lund et al., 2004; Sainz et al., 2004]. We also confirmed a higher antiviral effect with IFN-β than with IFN-α (Fig. 4 and Table II).

There are two distinct results subsequent to suppression of IFN signaling pathway by RSV infection. Young et al. [2000], reported that RSV is able to circumvent the anti-viral functions of IFN and replicate in human cells that produce and respond to IFN without blocking of IFN signaling. In contrast, Ramaswamy et al., noted that RSV acts on epithelial cells derived from airway to modulate (inhibit) IFN signal transduction. This effect is likely mediated through proteasome-dependent degradation of STAT2 [Ramaswamy et al., 2004]. Our results showed that induction of ISGs is not suppressed by RSV infection (Fig. 2), suggesting that RSV does not inhibit IFN signaling pathway. Our data in this experiment supported the result reported by Young et al. However, the reason for the discrepancy with Ramaswamy et al.'s findings is not known.

Overwhelming immune responses are believed to contribute to the progression of SARS, however little is known about proinflammatory cytokine dysregulation and the clinical progression of SARS. In this study, we measured the induction levels of several cytokine mRNAs in Caco2 cells infected with SARS-CoV, RSV, FluAV, or hPIV2. Increased expression of IL-6 and RANTES was found in SARS-CoV and RSV infections, but not in FluAV or hPIV2. Overexpression of IL-6 was reported in patients with SARS-CoV and RSV [Hornsleth et al., 1998; Zhang et al., 2004]. RSV infection causes more severe respiratory symptoms compared to infections with FluAV and hPIV2, and the serum concentration levels of IL-6, IL-8, and RANTES correlate with the symptom scores [Sung et al., 2001; Gern et al., 2002]. IL-8 and RANTES, potent neutrophil attractant and activator, has been shown to be elevated in blood and alveolar spaces [Chollet-Martin et al., 1996] and exhibit a positive correlation with the number of these chemokine in patients with pneumonia and acute respiratory distress syndrome [Villard et al., 1995]. Therefore, these chemokines induced by SARS-CoV may also play role in the accumulation of hemophagocytosis in the lung and development of subsequent wheezing

after SARS-CoV infection, and contribute to symptom severity of SARS.

Collectively, SARS-CoV infection significantly induces inflammatory cytokines and chemokines as well as RSV infection. This is likely what contributes to the onset of severe respiratory symptoms as compared with FluAV and hPIV2 infection. However, clinical signs or symptoms of SARS-CoV infection are more "severe" than those of RSV infection. What differences are there in inflammatory responses between SARS-CoV and RSV? We found a difference in induction of a negative regulator of cytokine signaling, SOCS family. The induced level of SOCS3 mRNA during SARS-CoV infection was clearly lower than that in RSV infection. IL-6 transcriptionally activates various genes contributing to inflammatory responses. The negative factor SOCS3 participates in the feedback system of IL-6 signal transduction by binding to phosphorylated tyrosine residue of a component of IL-6 receptor gp130 [Yasukawa et al., 2003]. Therefore, suppressed SOCS3 expression in SARS-CoV infected Caco2 cells might lead to continuous activation of STAT3, and prolonged and enhanced IL-6 signaling. Less induction of SOCS3 contributes to dysregulation of inflammatory signaling and increases the severity of inflammation in SARS-CoV infection.

Stimulation of cells with IL-6 leads to the activation of JAK/STAT, p38 mitogen-activated protein kinase (MAPK), AP-1 and Akt signal transduction pathways [Yang et al., 2003]. IL-6 dependent expression of SOCS3 is promoted by STAT3 activation through JAK/STAT signaling pathway [Lang et al., 2003]. Mizutani et al. [2004a], reported that SARS-CoV induced dephosphorylation of constitutive phosphorylated STAT3, resulting in the dysfunction of STAT3 transcriptional activity. The inactivation of STAT3 may contribute to less induction of SOCS3 in SARS-CoV infection. It is, therefore, important to investigate the influence of SARS-CoV on phosphorylation/dephosphorylation of STAT3. In addition, there is a possibility of a direct affect on the promoter region of SOCS3 gene in SARS-CoV infection. On the other hand, activation of p38 MAPK, AP-1 and Akt signal transduction pathway by SARS-CoV [Mizutani et al., 2004b,c] may be correlated with inflammatory responses.

In contrast to SARS-CoV, we recognized abundant induction of SOCS3 in RSV infection. Downregulation of appropriate cytokine signaling by feedback regulators, SOCS families, is thought to play an important role in the balance of cytokine signaling, thus contributing to the onset of Th1 and Th2 mediated immune response. Expression of SOCS3 correlates with the pathology of Th2 mediated allergic immune diseases such as atopic dermatitis and asthma [Seki et al., 2003]. Many epidemiological studies indicate possible linkages between RSV infection in childhood and subsequent manifestations of atopy and asthma [Sigurs et al., 2000; Holt and Sly, 2002]. The strong induction of SOCS3 by RSV could be a causative factor of atopy and asthma. Studies are underway to investigate the correlation between SOCS3 induction and cytokine regulation during RSV infection in FL and A549 cells, because we cannot detect SOCS3 protein in Caco2 cells infected with RSV and treated with IL-6. Difficulty of protein detection (SOCS3) is thought to be dependent on cell types, but not on SARS-CoV. In addition, other members of SOCS family, SOCS1 and CIS were also investigated in these viruses infected Caco2 cells. All of the viruses failed to express SOCS1, and only RSV was able to induce CIS (Fig. 5). Therefore, SOCS3 and CIS play an important role in inflammatory system in RSV infection.

Increased expression of TLR4 and TLR9 were found in SARS-CoV and RSV infections. The enhanced interaction between TLRs and some microbial substances has the potential to profoundly alter the grade of inflammation. Indeed upregulated TLR4 by RSV infection leads to increased binding of LPS to airway epithelium and enhanced inflammatory reaction [Monick et al., 2003]. Therefore, it is suggested that SARS-CoV and RSV infections enhance epithelial inflammatory response to some inhalant microbial substances by upregulated TLRs.

In conclusion, we consider that the overwhelming immune response of SARS results from dysregulation of the cytokine network caused by the overexpression of inflammatory cytokines and downregulation of feedback regulators. Our study will be a useful to help clarification of the mechanism of clinical progression of SARS by the cytokine regulation system and may lead to the establishment of a new therapeutic target in SARS.

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