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# The macrophage in the pathogenesis of severe acute respiratory syndrome coronavirus infection

## Key Messages

1. Severe acute respiratory syndrome (SARS) coronavirus (SCoV) initiates virus replication in macrophages with the production of negative sense viral ribonucleic acid and viral protein, but such viral replication is abortive and no infectious virus is produced.
2. In contrast to influenza A virus, infection with SCoV fails to induce type-1 interferons (eg IFN-β), which are key mediators of innate immune defence, but does lead to a strong induction of proinflammatory chemokines (eg IP10).
3. Sub-neutralising antibody to SCoV does not lead to the mediation of antibody-dependent enhancement of SCoV viral replication in macrophages.
4. Microarray analysis of SCoV infected cells identifies novel pathways including cytokines and chemokines and apoptotic pathways that are differentially activated by SCoV, which may be important in the pathogenesis of SARS, and deserve further studies.

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## Introduction

A novel coronavirus was identified as the causative agent of severe acute respiratory syndrome (SARS).[1] Compared with common respiratory viral infections, SARS is unusually severe, with an overall fatality rate of about 10%. The SARS coronavirus (SCoV) causes a range of disease from flu-like symptoms and viral pneumonia to acute respiratory distress syndrome and death. The previously known human coronaviruses 229E (HCoV-229E) and OC43 have only been linked with the common cold. However, several animal coronaviruses have resulted in severe animals diseases of the respiratory or gastrointestinal tract or disseminated infections.

Macrophages are key cells for host defence and are abundant within all tissues of the body, including the respiratory system. They are potent producers of cytokines that are crucial components of innate immunity and potential mediators of immunopathology. Genetic resistance to strains of the coronavirus mouse hepatitis virus is associated with the ability of the virus to replicate in macrophages.[2] In contrast, feline infectious peritonitis is a disease caused by a coronavirus in which prior immunity or passive antibodies increase the severity of the disease.[3] In this disease, macrophages are the main target cells for virus replication, and antiviral antibodies enhance the replication of the virus in macrophage cultures in vitro. This has led to concerns about whether antibody-mediated enhancement of disease may be relevant to the pathogenesis of SARS.

## Aims and objectives

1. Establish an in vitro model of SCoV infection of human primary macrophages;
2. Define the gene expression profile of SCoV-infected macrophages and compare it with human coronavirus 229E and influenza A (H1N1); and
3. Define the effect of antibody on neutralisation or enhancement of virus entry and replication.

## Methods

This study was conducted from June 2005 to November 2006.

### *In vitro model of SCoV infection in macrophages and gene expression profiling*

Using microarray gene expression profiling, we compared host response of primary human macrophages to infection with SCoV (strain HK39849), HCoV-229E, and influenza A virus (A/HK/54/98). The study was performed using macrophages derived from peripheral blood mononuclear cells of three different donors. The cells of each donor were subjected to microarray analysis after infection with each virus for 1, 3, and 6 hours. Ribonucleic acid (RNA) extracted from each macrophage preparation was examined for human genome-wide gene expression with a GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, USA) by the use of oligonucleotide probe sets, which spread across the full length of each gene in order to interrogate 28 869 genes (Genome Research Centre, The University of Hong Kong). Microarray data was normalised using

an ExonRMA summarisation algorithm on probe sets and baseline transform to a medium of all samples using the GeneSpring GX 9.0.5 software. By performing principal components analysis looking for outlier samples falling distal to the dataset at large and using filters on flags, quality control of sample levels was attained. Statistical analysis entailed a 2-way ANOVA test with a P-value cut-off of <0.05. Differential expression of genes to corresponding mock entities was selected with fold change of ≥1.5. Genes of individual pathways of interest were further studied using quantitative real-time polymerase chain reaction (RT-PCR) methods. Protein levels of key mediators were confirmed using enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions. Culture supernatants were ultraviolet-irradiated for 20 min to inactivate infectious viruses prior to assay in a biosafety level-3 facility. Previous experiments have confirmed that cytokine levels are not affected by the dose of ultraviolet radiation used.[4]

### *Immune enhancement assay of SCoV replication in macrophages*

We investigated the effect a human monoclonal (CR3014, a gift from Crucell Holland BV, Leiden, The Netherlands) and polyclonal (convalescent SARS serum) antibody to SCoV on the entry and replication of SCoV in human macrophages. Serial dilutions of the respective antibody or relevant control serum were mixed with a fixed dose of SCoV and infected onto human macrophages. Samples of the culture supernatants were collected at days 0, 1, 2, 3, 5, and 7 post-infection and titrated for virus infectivity. Ribonucleic acid was isolated from infected macrophages at 6 and 24 hours post-infection using the RNeasy Mini kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. Quantification of positive- and negative-strand viral RNA was performed by quantitative RT-PCR targeting the ORF1b gene, as described previously.[5] The SCoV RNA levels were normalised for the levels of β-actin mRNA.

## Results

### *SCoV infection of macrophages*

After infection of macrophages with SCoV, there was an increase in the copy numbers of both the positive and negative RNA strands of the SCoV ORF-1b and nucleocapsid genes over the first few hours after infection. Viral RNA levels in macrophages peaked at modest levels at about 6 h post-infection, but in FRhK-4 cells they continued to increase, reaching much higher absolute levels. A mouse monoclonal antibody (4D11) was used to demonstrate nucleoprotein expression in SCoV-infected macrophages. More than 90% of macrophages showed nucleoprotein expression when infected at a multiplicity of infection of one to two. However, no infectious virus was detected in the supernatant of virus-infected macrophages for up to 7 days post-infection, indicating that virus infection of these cells was abortive. In contrast, virus-infected FRhK-4 cells produced infectious virus titres up to $10^5$ in 50% tissue culture infective doses/ml (data not shown), peaking at about 2 to 3 days post-infection.

### *Microarray analysis*

In order to identify host genes that are affected by SCoV and thus account for its virulence, we compared the gene expression profile of SCoV-infected macrophages with that of low pathogenic viruses (influenza H1N1 and HCoV 229E) at various time points post-infection. Figure 1 illustrates the number of genes differentially affected by SCoV infection in comparison with influenza A or HCoV 229E. The proportion of genes uniquely affected by SCoV in comparison to H1N1 or HCoV 229E or both remained consistent at 77% at 1 and 3 h post-infection, but was down to 35% at 6 h post-infection. Figure 2 summarises selected microarray data presented as fold-change of gene expression in comparison to mock infected cells for innate immune markers at 1, 3 and 6 hours post-infection with SCoV, HCoV229E, and influenza A H1N1. Notably, IFN-β and -α-1 induction appears delayed or absent in SCoV- or HCoV 229E-infected macrophages. IP-10 induction is strongly induced at 1 and 3 hours by SCoV, while IL-8 is differentially down-regulated by SCoV (Fig 3). Apoptotic and anti-apoptotic pathways were also differentially activated in SCoV infected macrophages (data not shown).

Microarray analysis also suggests that other proinflammatory cytokines such as TNF, CCL2/MCP-1, CXCL10/IP-10 were strongly induced. Quantitative RT-PCR analysis confirmed an early induction of several chemokines, such as CXCL10/IP-10 and CCL2/MCP-1, in SCoV-infected macrophages. The ELISAs for CXCL10/IP-10 and CCL2/MCP-1 in macrophage culture supernatants

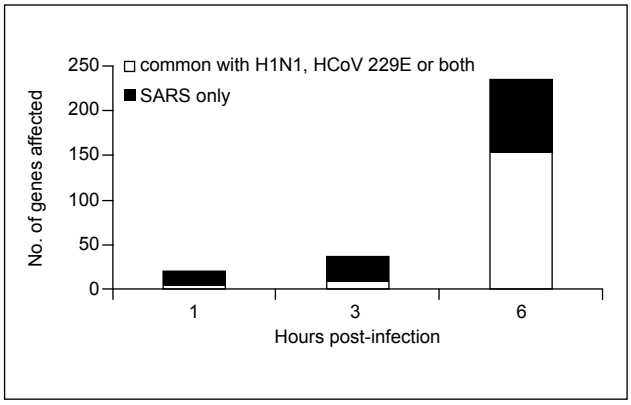


**Fig 1. Microarray analysis of host genes affected by SCoV-infected macrophages**

Gene expression profile of SCoV-infected, primary monocyte–derived macrophages is compared with that of H1N1 and HCoV 229E infections at 1, 3, and 6 hours post-infection. Genes with level change of 1.5 folds are regarded as affected. Genes that are affected by SCoV infection as well as by H1N1, HCoV 229E, or both are shown (white bar), with the proportion of the genes that are affected by SCoV indicated (black bar)

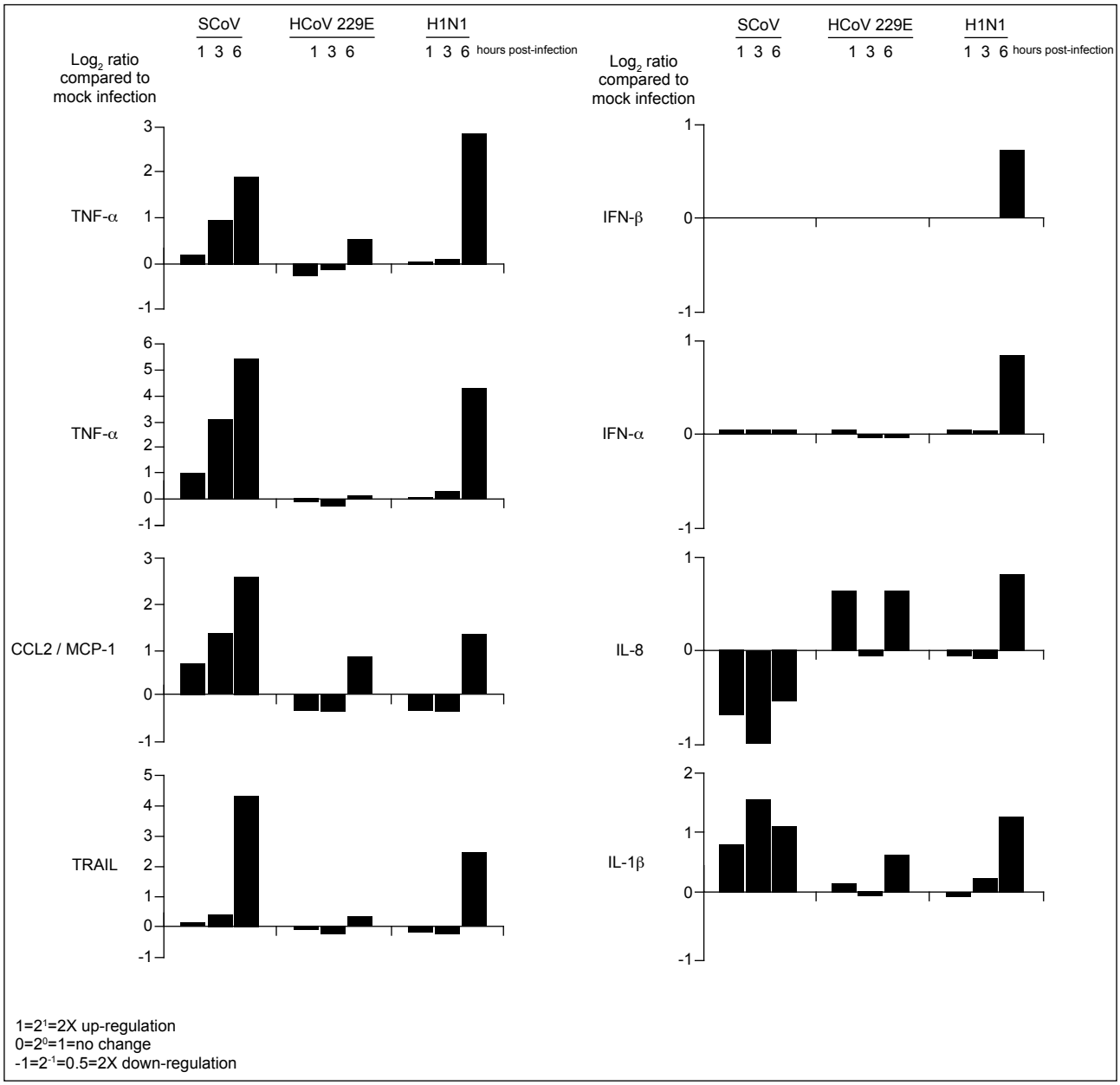


**Fig 2. Microarray data showing fold change in gene expression of selected innate immune response genes differentially affected by SCoV-infected macrophages**

Gene expression profile of SCoV-infected, primary monocyte–derived macrophages is compared with that of H1N1 and HCoV 229E infections at 1, 3, and 6 hours post-infection. Composite data from three different donors is presented. Gene expression is denoted with reference to that in mock infected cells

confirmed that SCoV induced CXCL10/IP-10 and CCL2/MCP-1 secretion in macrophages in the first few hours after infection.

In collaboration with a research group showing that SCoV Orf3a has apoptotic activity, we investigated the function of SCoV3a in SCoV-infected cells. The SCoV3a was localised to the Golgi region and interacted with caveolin 1 (according to yeast two hybrid analysis). There was evidence that caveolin 1 may be found in SCoV particles (data not shown). This is relevant to the microarray data where SCoV differentially activated a number of pro- and anti-apoptotic genes.

### *SCoV does not replicate in human macrophages in the presence of mAb CR3014 or convalescent serum*

The SCoV did not replicate in primary human macrophages to produce infectious virus at measurable titres and the addition of serial dilutions of a human monoclonal antibody to SARS CoV spike (mAb CR3014). Serum from a convalescent SARS patient did not convert this abortive infection to a productive one. Productive virus replication was assayed by cell culture titration and by strand-specific RT-PCR assays to detect the negative sense (replication

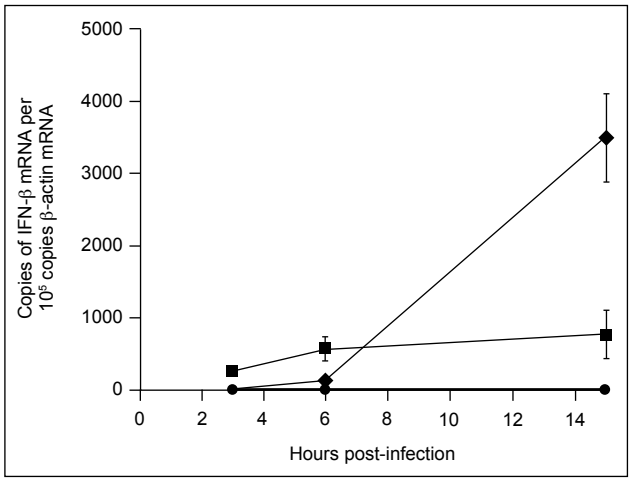


**Fig 3 (a). Lack of induction of IFN-ß gene expression in SCoV-infected macrophages**

Levels of IFN-ß mRNA are determined by quantitative real-time polymerase chain reaction. Macrophages are infected with SCoV (●), HCoV-229E (◆), and influenza A (H1N1) virus (■) at a multiplicity of infection of one to two, and RNA is extracted at 3, 6, and 15 h post-infection. The SCoV-infected macrophages does not induce IFN-ß at any of the three time points, in contrast to infections with influenza A (H1N1) and HCoV-299E viruses

intermediate) and positive sense viral RNA. With or without monoclonal or polyclonal antibody, macrophages took up SCoV, but this uptake did not lead to the productive virus replication and release of infectious virus.

## Discussion

This study confirmed that virus gene transcription and translation were initiated in infected macrophages and that the block in productive virus replication occurred subsequently. Although double-stranded RNAs (which are potent inducers of type-1 interferon) and viral protein were expressed in SCoV-infected macrophages, there was no detectable IFN-β response in these cells. Others have reported similar findings in other cell types (eg epithelial).[6] The SCoV also failed to induce IL-28 and -29, which are two other recently discovered interferon-like cytokines with antiviral activities.[7] In contrast, both HCoV-229E and influenza A virus induced IFN-β as well as IL-28 and IL-29 in macrophages, although such induction was delayed in HCoV 229E in comparison with influenza A. This lack of innate immune defences may explain the progressive

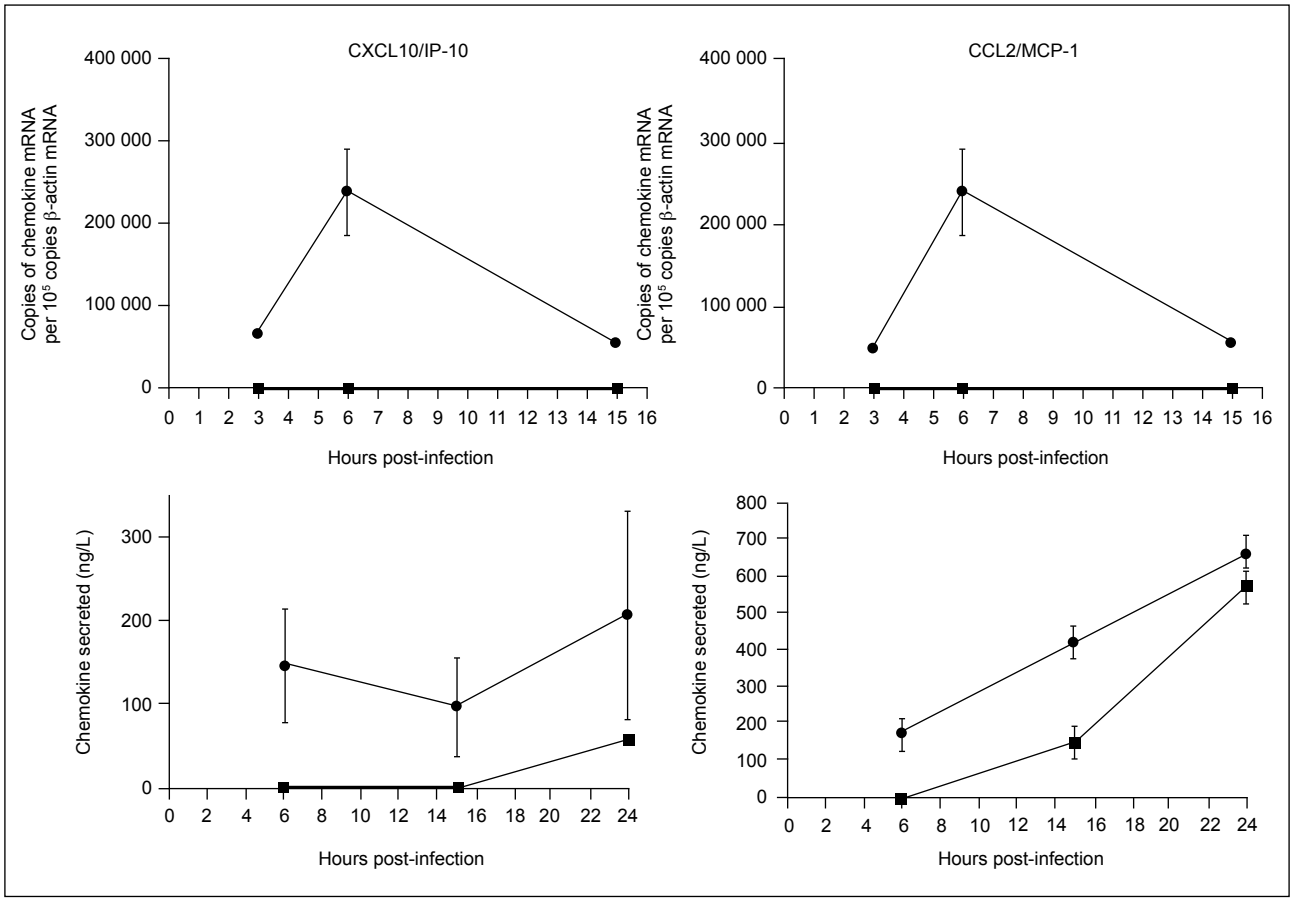


**Fig 3 (b). Levels of CXCL10/IP-10 and CCL2/MCP-1 are elevated in SCoV-infected macrophages**

Macrophages are infected with SCoV at a multiplicity of infection of one to two. Ribonucleic acid is extracted at 3, 6, and 15 h post-infection, and the levels of mRNA for CXCL10/IP-10 and CCL2/MCP-1 are determined by quantitative real-time polymerase chain reaction (top). Aliquots of the culture supernatant are taken at 6, 15, and 24 h post-infection, and the levels of secreted CXCL10/IP-10 and CCL2/MCP-1 are determined by specific enzyme-linked immunosorbent assays (bottom). The means±standard deviations of duplicate cultures from the same donor are representative of three independent experiments with similar results. SCoV infection (●) of macrophages induces higher levels of gene expression and secretion of CXCL10/IP-10 and CCL2/MCP-1 than does mock infection (■)

increase of viral load in the nasopharyngeal secretions up to week 2 of SARS infection, in contrast to other respiratory infections such as HCoV-229E and influenza A virus.[8]

The SCoV protein nsp1 has been identified as a putative interferon antagonist but its signalling pathways are unclear.

Chemokines such as CXCL10/IP-10 and CCL2/MCP-1 were up-regulated in macrophages by SCoV. CXCL10/IP-10 and CCL2/MCP-1 are chemotactic for monocytes/macrophages, which are the predominant inflammatory cell type in the lungs of SARS patients. We (unpublished data) and others[9] have found significantly elevated blood levels of CXCL10/IP-10 and CCL2/MCP-1 in SARS patients and that both chemokines were significantly elevated during the early stage of the illness. The chemokines CCL3/macrophage inflammatory protein 1, CCL7/MCP-3, and CCL8/MCP-2 were induced by SCoV according to the microarray analysis, and their biological effects were similar to CCL2/MCP-1. Therefore, the members of the monocyte chemotactic protein and macrophage inflammatory protein can synergistically induce a cycle of monocyte/macrophage recruitment and, potentially, monocyte/macrophage-induced immunopathology.

In this study, we also addressed the potential problem of antibody-dependent enhancement (ADE), which is a well-recognised phenomenon observed in infections with other coronavirus—feline infectious peritonitis virus. Given that ADE in feline infectious peritonitis virus infection is mediated by increased macrophage uptake of virus in the presence of neutralising antibody, we performed human macrophage infectivity assays in the presence of serial dilutions of CR3014 and human convalescent serum. The addition of varying concentrations of CR3014 or convalescent SARS serum to SCoV did not convert the abortive infection into a productive one. This reduced the likelihood that ADE in macrophages will be observed in vivo after passive immunisation in a manner analogous to that with feline infectious peritonitis.

## Conclusions

The lack of a type-1 interferon response despite a strong induction of macrophage tropic chemokines may explain aspects of the pathogenesis of SARS. Although putative viral proteins such as the nsp1 have been implicated as interferon antagonists in SCoV, the signalling mechanisms that underlie this suppression of interferon remain unknown and deserve further research. The apparent inability of SCoV to trigger interferon responses may provide support for the use of interferon treatment for SARS.

## Acknowledgements

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