

# Antibody-dependent enhancement of SARS coronavirus infection and its role in the pathogenesis of SARS

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## KEY MESSAGES

1. Anti-SARS-CoV spike antibodies promote infection of primary human immune cells by SARS-CoV.
2. The antibody-dependent enhancement (ADE) infection pathway grants SARS-CoV an opportunity to infect primary human macrophages, but it does not sustain productive viral replication in the infected cells.
3. ADE of SARS-CoV infection does not alter pro-inflammatory gene expression profile of primary human macrophages.
4. Infectivity of SARS-CoV does not rely solely on the potency of target cells to bind - via Fcγ receptor II (CD32) - infectious immune complexes, but depends on the properties of the intracellular domain of the FcγRII.
5. Occurrence of ADE of SARS-CoV infection into

human primary macrophages, without alteration to their pro-inflammatory properties, advocates cautious development of SARS-CoV vaccine in humans, and provides new ways of investigation to understand the pathogenesis of SARS.

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## Introduction

Infection with SARS-CoV involves not only the respiratory tract but also the gastrointestinal tract and other organ systems. Several reports have highlighted the direct infection of haematopoietic cells by SARS-CoV. It is unclear how the virus gets into immune cells that do not express the SARS-CoV receptor angiotensin I converting enzyme 2 (ACE2).

Immune-mediated infections and, in particular, antibody-dependent enhancement (ADE) is known to be exploited by a variety of viruses, such as dengue virus, HIV, and animal coronavirus, as an alternative way to infect host cells.<sup>1</sup> In addition to an interaction between viral protein and host receptors, these viruses can enter cells through the binding of virus/antibody immune-complexes to Fc receptors (FcR), complement receptors, or alternatively by inducing conformational change in envelope glycoproteins that are required for virus-cell membrane fusion.<sup>1</sup>

We have demonstrated that anti-spike antibodies potentiate infection of both monocytic and lymphoid human immune cell lines with SARS-CoV Spike-pseudotyped lentiviral particles (recombinant viruses encoding a reporter gene and bearing SARS-CoV Spike proteins at the virion surface; SARS-CoVpp) and also with replication-competent SARS-coronavirus.<sup>2</sup> Because antibody-

mediated infection accounts for the altered response to infection, we hypothesised that ADE of SARS-CoV infection—by widening SARS-CoV tropism toward immune cells—would elicit a hyper-induced profile of immune mediators (cytokine/chemokines) that would impair the homeostasis of the immune system and ultimately contribute to SARS pathogenesis.

This study highlighted the occurrence of ADE of SARS-CoVpp infection in different circulating immune cell types, among which the monocytic lineage was a prime target. In addition to monocytes, infection of human macrophages was enhanced in the presence of anti-viral antibodies. Despite being increasingly susceptible to infection, macrophages did not support productive replication of the virus, or modify expression of some pro-inflammatory cytokines/chemokines upon antibody-mediated invasion. Our findings point towards the likely occurrence of ADE infection of immune cells by SARS-CoV, but the outcomes of such an alternative infection pathway on the cell functionality/homeostasis remain unclear.

## Methods

This study was conducted from October 2009 to September 2011.

## Cell lines and primary human immune cell cultures

VeroE6 (African green monkey kidney epithelial cells), Raji (Burkitt's lymphoma/B lymphoblast), parental ST486 (Burkitt's lymphoma/B lymphoblast lacking expression of FcγR) and FcγR/ST486 were cultured as previously described.<sup>2</sup>

Peripheral blood mononuclear cells (PBMCs) were prepared from human buffy coats of healthy blood donors (Hong Kong Red Cross Blood Transfusion Service). The research protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW09-375). Monocyte-derived macrophages were generated *in vitro* using standard protocol. Briefly, mononuclear cells were isolated by a Ficoll-Paque density gradient (Pharmacia Biotech) and monocytes were enriched by plastic adherence. Alternatively, CD14<sup>+</sup> purified mononuclear cells were prepared by positive magnetic bead selection (Miltenyi biotec). Cells were then seeded on tissue culture plates in RPMI 1640 medium supplemented with 5% heat-inactivated autologous plasma, 0.6 mg/L penicillin and 60 mg/L streptomycin at 10<sup>6</sup> cells/mL (Life Technologies). The cells were replenished with fresh medium every 2 days and allowed to differentiate for 14 days. The purity of macrophages was consistently above 90%, as ascertained by immunofluorescence staining for human CD68 (macrosialin/gp110). Depending on the downstream application, macrophages were either seeded on a glass-cover slip or in an appropriate culture vessel the day before infection. All cells were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> supply.

## Immunisation with recombinant spike proteins or inactivated SARS-CoV

Recombinant SARS-CoV spike protein production and immunisation procedure have been described.<sup>2</sup> Six- to 8-week-old BALB/c mice (n=4-5 per group) were immunised intraperitoneally at 3-week intervals with 2 μg of FLAG-tagged recombinant codon-optimised SARS-CoV spike full-length or injected with saline solution emulsified with 1 mg of alum. Sera were collected at day 55 post-immunisation, heat-inactivated for 30 minutes at 56°C and stored at -20°C for subsequent use.

## Production and use of lentiviral pseudotyped particles

The pseudotyped viral particles expressing a luciferase reporter gene were produced as described.<sup>2</sup> Briefly, SARS-CoV spike pseudotyped lentiviral particles (SARS-CoVpp), vesicular stomatitis virus glycoprotein pseudotyped lentiviral particles (VSVpp) or lentiviral particles lacking

expression of any viral envelope protein (Δenvpp) were obtained by transfection of HEK293T cells with an HIV-1 provirus construction (pNL4.3.Luc R-E<sup>pro</sup>) and a plasmid encoding the viral envelope protein of interest, ie SARS-CoV spike, VSV-G, or empty vector (pcDNA3.1; Invitrogen), respectively. Following a purification step on 20% sucrose cushion, the concentrated viral particles were titrated by ELISA for lentivirus-associated HIV-1 p24 protein according to the manufacturer's instructions (Cell Biolabs) and the viral stocks were stored at -80°C until use.

## Infection with SARS-CoV

Serial, 2-fold dilutions of heat-inactivated mouse serum were incubated for 1 hour at 37°C with an equal volume of live SARS-CoV (strain HKU-39849) under appropriate containment in a BSL3 laboratory (Department of Microbiology, The University of Hong Kong). Cells were infected at a multiplicity of infection (MOI) of 1 for 60 minutes at 37°C, washed and then incubated in supplemented culture medium containing appropriate dilutions of mouse serum. At the end of the experiment cells were either fixed in 4% paraformaldehyde (dissolved in phosphate-buffered saline) for immunofluorescence microscopy, or resuspended in lysis buffer (RLT buffer, RNeasy RNA Mini kit; QIAGEN) for real-time quantitative PCR and stored appropriately until use. Additionally, samples of cell culture supernatants (100 μL) harvested at different time points were mixed with 350 μL of RLT buffer and stored at -80°C until use.

## Immunofluorescence microscopy

To assess SARS-CoV infection, cells were incubated for 45 minutes with a mouse monoclonal antibody specific for the viral nucleoprotein (N), and revealed by secondary TRITC-conjugated goat anti-mouse (Zymed Laboratories). To assess SARS-CoV Spike pseudoparticle (SARS-CoVpp) infection, cells were incubated for 45 minutes with FITC-conjugated polyclonal goat anti FireFly luciferase (Rockland). Slides were assembled with DAPI-containing mounting reagent (Southern Biotech) and analysed with an AxioObserver Z1 microscope (Zeiss). Pictures from 5-10 randomly selected fields were acquired with an Axiocam MRm camera and processed with MetaMorph software (Molecular Devices).

## Real-time quantitative RT-PCR for viral gene expression

Total RNAs were extracted with an RNeasy RNA Mini kit (QIAGEN), with DNase digestion, according to the manufacturer's instructions. Extracted RNAs were stored at -80°C until use. Superscript III reverse transcriptase (Invitrogen)

and random hexamer primers (Invitrogen) or gene-specific oligonucleotides were used to convert RNAs to cDNAs. The amount of viral and host RNA was measured by real-time quantitative PCR using SybrGreen-based technology on a LightCycler 480-II instrument (Roche).

### Statistical analysis

Differences between groups were compared using the unpaired Student's t-test with a 0.05 significance level.

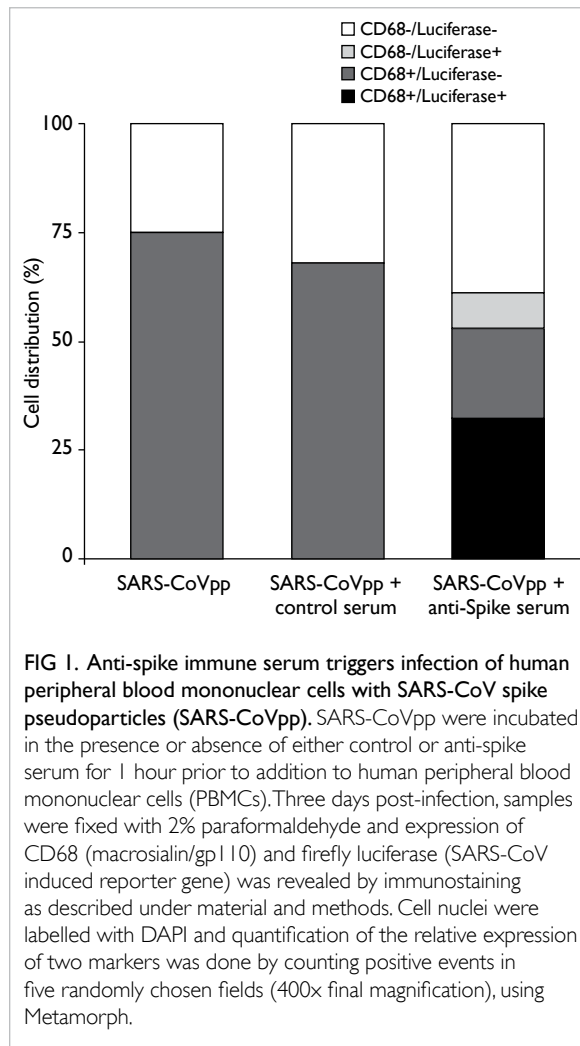
## Results

### Anti-spike immune serum promotes infection of primary human immune cells with SARS-CoV

Following previous observations made with human immune cell lines,<sup>2</sup> we determined whether ADE of SARS-CoV infection could occur also in primary human immune cells. To explore this hypothesis, we pre-incubated SARS-CoVpp with mouse anti-spike control serum prior to infection of PBMCs. We then examined the extent of infection by monitoring the expression of the luciferase reporter protein encoded by the SARS-CoVpp. Compared with controls, infection in the presence of heat-inactivated anti-spike serum facilitated infection of human PBMCs with SARS-CoVpp (Fig 1). In order to shed light on the identity of the ADE-infected primary immune cells, co-staining for the widely accepted myeloid marker CD68 (macrosialin/gp110) was also performed. Among the cells infected in the presence of anti-viral antibodies, over 75% were positive for CD68 (Fig 1). Because CD68 was expressed on a variety of cells other than the monocytic lineage, we highly purified CD14<sup>+</sup>-cells (ie monocytes) and assessed their susceptibility to ADE of SARS-CoVpp infection.

### The monocytic lineage is a prime target for ADE of SARS-CoV infection

As previously shown with the monocytic cell line THP-1,<sup>2</sup> primary human monocytes were susceptible to infection by SARS-CoVpp in the presence of anti-spike antibodies only. Inoculums made of SARS-CoVpp, or SARS-CoVpp pre-incubated in the presence of control serum never resulted in a detectable luciferase signal (not shown). Because in the course of the deadly feline infectious peritonitis, anti-spike antibodies have been shown to trigger massive infection of macrophages, a key event for disease pathogenesis, we also monitored occurrence of antibody-mediated SARS-CoV infection in human monocyte-derived macrophages. When human macrophages were infected in the presence of anti-spike immune-serum, detection of the luciferase reporter protein was markedly different compared



**FIG 1. Anti-spike immune serum triggers infection of human peripheral blood mononuclear cells with SARS-CoV spike pseudoparticles (SARS-CoVpp).** SARS-CoVpp were incubated in the presence or absence of either control or anti-spike serum for 1 hour prior to addition to human peripheral blood mononuclear cells (PBMCs). Three days post-infection, samples were fixed with 2% paraformaldehyde and expression of CD68 (macrosialin/gp110) and firefly luciferase (SARS-CoV induced reporter gene) was revealed by immunostaining as described under material and methods. Cell nuclei were labelled with DAPI and quantification of the relative expression of two markers was done by counting positive events in five randomly chosen fields (400x final magnification), using Metamorph.

with those infected in the presence of control serum (not shown). Of note, infection of primary macrophages with recombinant viral particles pseudotyped with the glycoprotein of the VSVpp or Δenv.pp was never modified by the presence of anti-SARS-CoV spike immune serum (not shown). These experiments indicate that anti-spike antibodies facilitate infection of SARS-CoVpp but not VSVpp or Δenv.pp into human primary macrophages.

### Antibody-mediated enhancement of SARS-CoV infection in primary macrophages leads to abortive infection

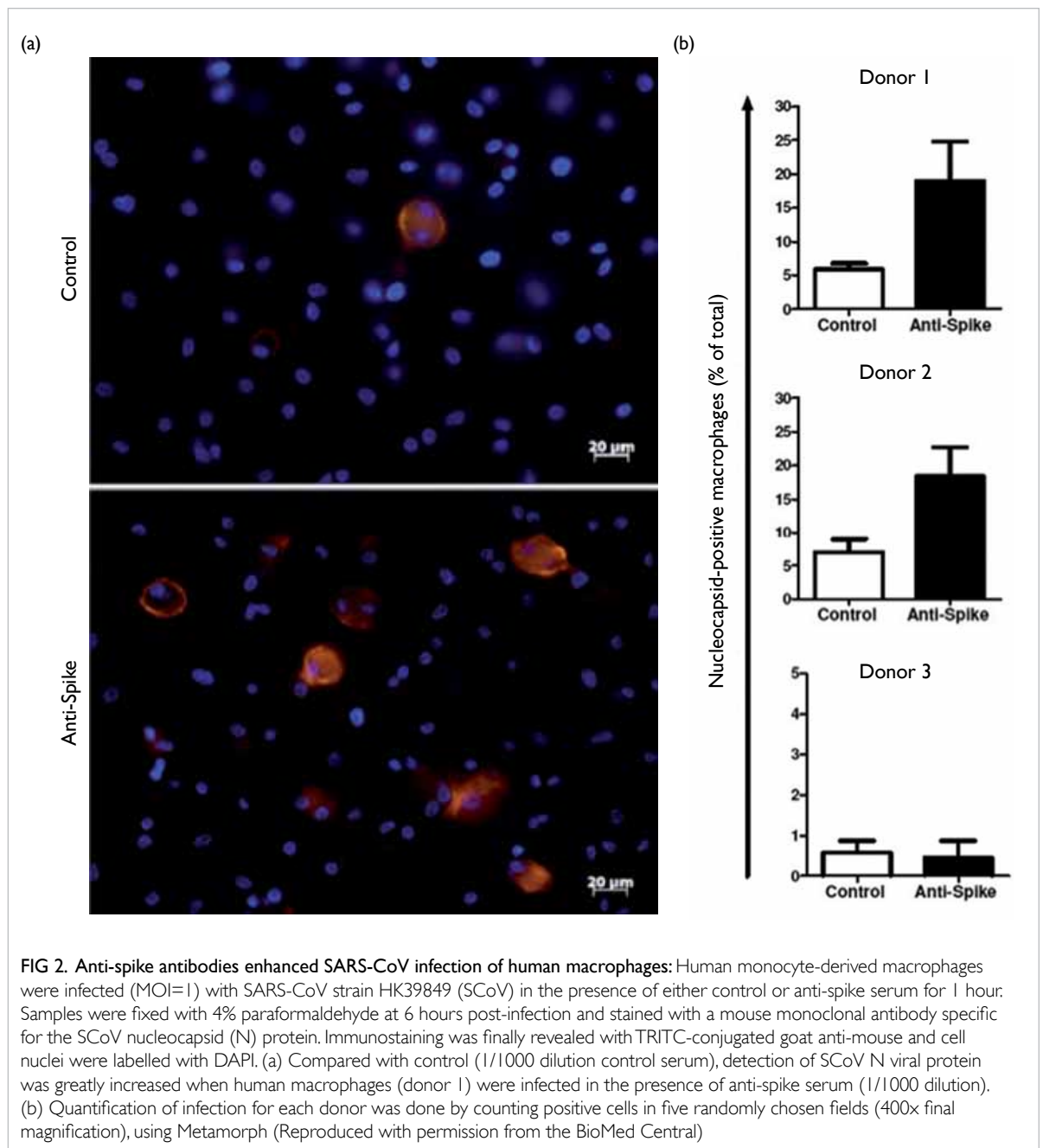
Because of the well-established roles of the macrophages in the primary response to pathogens and in the shaping of the subsequent (pathogen-specific) adaptive immune response, we investigated whether a change in tropism could also be observed during infection with replication-competent SARS-CoV.

Infection of monocyte-derived macrophages

by replication competent SARS-CoV in the presence of control serum led to modest infection. In contrast, when they were infected in the presence of anti-spike immune-serum, intracellular detection of viral nucleocapsid protein was markedly increased (Fig 2). To assess the capability of SARS-CoV to productively replicate into ADE-infected macrophage cells, we monitored the cellular viral load with real-time quantitative RT-PCR as well as the release of SARS-CoV progeny into cell culture supernatant.

Quantitative measurements of either positive (ie genomic and mRNA) or negative (ie subgenomic replicative intermediates) RNA strands

demonstrated a significant increased viral load at 6 hours post-infection in cells infected in the presence of anti-spike serum, followed by a continuing decrease in both viral RNA species with time so that no difference between groups was noted later on (not shown). Of note, there was no evidence of spreading infection to other cells (not shown). Similarly we did not detect any change in viral gene copies when we assessed the viral yield of macrophage-infected supernatants, whether incubated in the presence or absence of immune serum. Altogether these results indicate that despite the ability of SARS-CoV to exploit anti-viral antibodies to invade macrophages,



only abortive replication occurs upon infection and no infectious virus is released from the ADE-infected cells.

### **ADE infection pathway does not alter the profile of cytokine/chemokine produced by primary human macrophages**

The antibody-mediated entry route would affect functionality and cell homeostasis of ADE-infected macrophages but would not result in productive infection. Indeed, ADE infection induced the initiation of viral gene transcription, with production of viral gene intermediate species, and viral protein synthesis. Given the ability of intracellular innate immune sensors, such as the pattern recognition receptor families TLR and RLH, to detect viral gene species and the disturbance to the cell homeostasis caused by SARS-CoV viral proteins (such as 3a protein and SARS-Unique Domain), we evaluated the expression profile of some characteristic proinflammatory immune mediators and apoptosis-inducing ligands. As previously documented, infection of human monocyte-derived macrophages with SARS-CoV led to induction of several proinflammatory cytokines and chemokines, without expression of type-I interferon IFN- $\beta$  (not shown). Similarly, infection in the presence of control serum did not trigger induction of IFN- $\beta$  and similar kinetics and levels of TNF- $\alpha$ , CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , and CXCL10/IP-10 were detected (Fig 3). When cells were infected in the presence of anti-SARS-CoV spike immune-serum (Fig 3), despite a 3-4 fold increase in intracellular SARS-CoV viral load (Fig 2), no change in the kinetic or in the expression of the type-I interferons and the proinflammatory cytokines/chemokines was noted. Similarly, compared with controls ADE-infection of macrophages never altered gene expression of prototypical death ligands such as TNF- $\alpha$  (Fig 3), FasL or TNF-related apoptosis-inducing ligand (not shown). Our results indicated that despite an enhanced infection of cells in the presence of anti-viral antibodies, the occurrence of ADE of SARS-CoV entry had little effect on the inflammatory response and the death ligand-induced killing activity of the macrophages.

### **The occurrence of ADE of SARS-CoV infection relies on the intracellular signalling domain of the human Fc $\gamma$ R by the target cells**

Our previous work demonstrated a major role for immune receptors, particularly Fc-gamma receptor II (Fc $\gamma$ RII) family, in triggering infection of immune cells with SARS-CoV.<sup>2</sup> Because this FcR family includes members that bind immunoglobulins/immune complexes with different affinities in addition to delivering either activating or inhibitory signals, we established a model to understand what, from

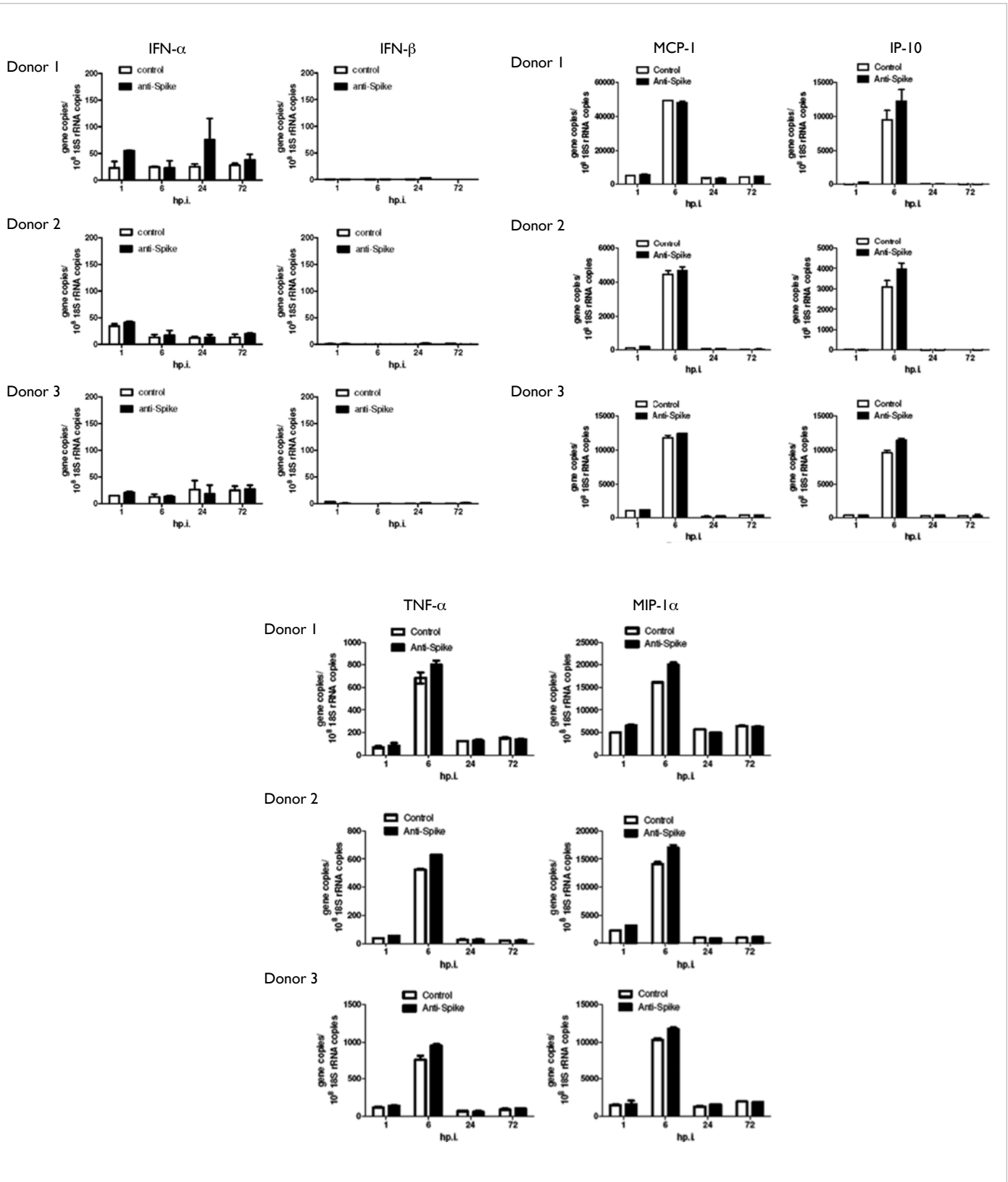
binding event to the Fc $\gamma$ RII or from internalisation/signalling cascade, played a preponderant role in the occurrence of ADE infection. Thanks to molecular mutagenesis, we established immune cell lines that expressed either a truncated form (ie lacking intracellular domain) or chimeric form (ie swapping of the intracellular domain) of Fc $\gamma$ RIIA (CD32a) and Fc $\gamma$ RIIB (CD32b), the only two receptors proved to be involved in ADE of SARS-CoVpp.<sup>2</sup> Our results indicated a prominent role of the intracellular domain over the extracellular domain of human Fc $\gamma$ RII in the occurrence of ADE of SARS-CoVpp.<sup>3</sup> All the Fc $\gamma$ RII receptors with truncation of their intracellular domain became unable to trigger ADE infection despite being able to efficiently bind immune complexes. This demonstrated that attachment of the immune-complexes to the target cells was not an event sufficient to trigger ADE of SARS-CoVpp infection (not shown).

## **Discussion**

The possibility that an immune response to pathogens may also have deleterious effects on the host homeostasis has been reported. For example, the hyper-induction of cytokines following avian influenza infection has been implicated in the severity of the disease, and infection of cells by antibody-dependent enhancement has been known to occur for several viral diseases.<sup>1</sup> In our study, anti-spike antibody potentiated infection of primary human immune cells with SARS spike-pseudotyped lentiviral particles and replication-competent SARS-coronavirus. Nonetheless in macrophages, antibody-mediated infection did not lead to productive replication of the virus, and did not induce a skewed profile of proinflammatory cytokines and chemokines.

After initiation of viral gene transcription and viral protein synthesis, a block seems to occur in the replication process ultimately ending in an abortive viral cycle without detectable release of progeny virus. Such abortive replication of SARS-CoV into macrophages has been documented,<sup>4</sup> but conversely to this previous report where 90% of the macrophages were infected with SARS-CoV in the absence of immune-serum (MOI=1-2). We detected a much lower infection yield (about 5-7%) using a similar experimental setup. Of note from three independent donors, two macrophage cultures showed evident infection ( $\geq 5\%$  infected macrophages; donor 1 and 2), whereas one displayed low permissiveness ( $< 1\%$  infected macrophages; donor 3) [Fig 2]. In the latter, addition of anti-spike antibodies did not make the macrophages more susceptible to infection, thus likely illustrating inter-individual variability of human macrophage to SARS-CoV infection.

Despite a four-fold increase in infection and initiation of viral gene transcription in the



**FIG 3. Induction of cytokines/chemokines by SARS-CoV in human macrophages following antibody-mediated infection:** Human monocyte-derived macrophages were incubated for 1 hour in the absence or presence of SARS-CoV strain HK39849 (SCoV), with two-fold serial dilutions (1/1000 and 1/2000) of either control (open bars) or anti-spike (closed black bars) serum. Cells were lysed at 1, 6, 24, and 72 hours post-infection, and total RNAs were extracted for RT-PCR amplification. The mRNA levels of different cytokines and chemokines were determined by real-time quantitative RT-PCR and for the sake of clarity only one serum dilution (1/1000) is shown. In most cases, when induced, cytokines/chemokines were detected before 24 hours post-infection only. Addition of anti-spike serum, compared with control serum, did not lead to a skewed expression profile and no significant changes were seen (Reproduced with permission from the BioMed Central)

ADE-infected macrophages, no change in gene expression of some pro-inflammatory cytokines/chemokines and death ligands was detected. Although we did not thoroughly investigate the occurrence of cell death with dedicated methods, our microscopic observations did not reveal chromatin condensation or membrane blebbing of the ADE-infected macrophages up to 72 hours post infection. Considering approximately 20% of macrophages were ADE-infected, and in regard to the apparent well-being of the infected cultures with cell loss/variation in total cell number of less than 8% throughout the 3-day assay, we speculate that in macrophages, ADE of SARS-CoV infection does not trigger massive cell death.

In regard to clinical observations of poor disease outcomes in early seroconverted SARS patients,<sup>5</sup> and because ADE of other viral infections (such as Ross River Virus, feline coronavirus, poliovirus, coxsackievirus) have been shown to elicit markedly distinct responses compared with viral entry through the natural host receptor, and as immune deregulation is a hallmark of SARS, involvement of ADE mechanisms during SARS pathogenesis was worthy of investigation.

The findings of this study partially invalidated our initial hypothesis that antibody-mediation would, by widening tropism of SARS-CoV toward immune cells, elicit an altered profile of immune mediators that impair homeostasis of the immune system and ultimately contribute to SARS pathogenesis. Nonetheless, it is still intriguing to us that triggering an enhanced infection of cornerstone innate immune cells such as macrophages would have no consequences. It is true that in contrast to studies that showed cell perturbation following ADE-infection, others have documented fewer, barely discernible consequences.<sup>6</sup> Hence it is possible that ADE of SARS-CoV infection is happening without deleterious consequences to the target cells. Nonetheless, with reference to the great deal of controversy in the literature regarding the relationship between immune mediators, cell death and the pathophysiological events of SARS, our study may not have focused on the right gene candidates. Thus for future investigations, it is advised to conduct genome-wide gene expression profiling.

## Conclusion

Our results suggest the rational development of vaccination strategies in the event of a SARS-like virus outbreak, with reasonable concerns about the occurrence of severe adverse effects.

## Acknowledgments

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