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Chemokine upregulation in SARS coronavirus infected human monocyte derived dendritic cells

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Abbreviations used in this paper:

SARS, severe acute respiratory syndrome; CoV, coronavirus; DC dendritic cell; CB, cord blood; FRhK-4 cells, fetal rhesus kidney 4 cells; MOI, multiplicity of infection

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Abstract

Lymphopenia and increasing viral load in the first 10 days of severe acute respiratory syndrome (SARS) suggested immune evasion by SARS-coronavirus (CoV). In this study, we focused on dendritic cells (DCs) which play important roles in linking the innate and adaptive immunity. SARS-CoV was shown to infect both immature and mature human monocyte derived DCs by electron microscopy and immunofluorescence. The detection of negative strands of SARS-CoV RNA in DCs suggested viral replication. However, no increase in viral RNA was observed. Using cytopathic assays, no increase in virus titre was detected in infected DCs and cell culture supernatant, confirming that virus replication was incomplete. No induction of apoptosis or maturation was detected in SARS-CoV infected DCs. The SARS-CoV infected DCs showed low expression of antiviral cytokines (IFN-α, IFN-β, IFN–γ and IL-12p40), moderate upregulation of proinflammatory cytokines (TNF-α and IL-6) but significant upregulation of inflammatory chemokines (MIP-1α, RANTES, IP-10 and MCP-1). The lack of antiviral cytokine response against a background of intense chemokine upregulation could represent a mechanism of immune evasion by SARS-CoV.

Introduction

Coronaviruses (CoV) comprise a large family of RNA viruses that infect a broad range of vertebrates, from mammalian to avian species.[1] Prior to the emergence of severe acute respiratory syndrome (SARS) in 2002-2003, human CoV were known to be associated mainly with relatively mild upper respiratory diseases such as the common cold. The novel SARS-CoV, however, caused severe, rapidly progressive atypical pneumonia with fever, myalgia and diarrhoea.[2,3] The detection of virus in stool and urine in addition to the respiratory tract of SARS patients further suggested that SARS is a systemic disease.[4,5]

At autopsy, white pulp atrophy was observed in the spleen and there was lymphoid depletion in lymph nodes.[6-8] Together with lymphopenia and increasing viral load in the first 10 days of disease,[3,4,6] these clinical features strongly suggest an evasion of the immune system by SARS-CoV. As with other viral infections, such as measles, this lymphoid depletion may have pathogenic significance.

Dendritic cells (DCs) are antigen presenting cells which play key roles in linking innate and adaptive immunity.[9-11] Immature DCs reside in the respiratory tract for immune surveillance and they respond dynamically to local tissue inflammation in the airways and the distal lung.[12,13] They express a wide range of receptors, including c-type lectins[14,15] and toll-like receptors,[16,17] for the recognition of conserved pathogen patterns. Dendritic cells signal the presence of danger to cells of the adaptive immune response and modulate their responses via the secretion of proinflammatory and/or antiviral cytokines.[18] In particular, DCs secrete cytokines to polarize T-helper (Th) cells towards the Th1 or Th2 subsets.[10]

The migration of DCs from tissues to lymph nodes is essential for antigen presentation and triggering of adaptive immune responses. The trafficking of DCs is regulated by chemokines which can be classified as homeostatic (constitutively expressed) or

inflammatory (induced/augmented) according to their immune fuctions.[19-21] Acute respiratory viruses commonly induce inflammatory chemokines, such as macrophage inflammatory protein (MIP) – 1α, regulated upon activation, normal T cell expressed and secreted (RANTES), interferon-inducible protein of 10kD (IP-10) and monocyte chemotactic protein (MCP)-1, in local tissues.[21] Dendritic cells are also a major source of these chemokines.[20]

Based on the function of DCs in immune surveillance, priming and tolerance, we hypothesized that DCs play an important role in the immunopathology of SARS. In addition, the developmental status of the host immune cells may affect their responses to viral infection. Hence, we also compared the cytokine and chemokine gene expression in SARS-CoV infected adult and cord blood (CB) DCs. This study provides evidence that SARS-CoV can infect DCs and alter their cytokines/chemokines production. Our results suggest possible mechanisms of immune escape and amplification of immunopathology in SARS.

Materials and method

Samples

Adult blood samples were from the white cell fraction of blood donated to the Hong Kong Red Cross by normal healthy volunteers. Human umbilical cord blood (CB) samples were collected from the placenta of normal full-term uncomplicated pregnancies. Informed consent was obtained from the mothers prior to delivery. The protocol was approved by the Institutional Review Board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster [EC1473-00].

Cell separation

Blood mononuclear cells were isolated from whole blood by centrifugation, using Ficoll-Hypaque gradients (Pharmacia Biotech, Uppsala, Sweden), washed, and labeled with immunomagnetic antibodies. Positive selection was performed according to manufacturer's specification (Miltenyi Biotec, Bergisch Gladbach, Germany) as in previous experiments.[22-26] Isolated CD14+ monocytes from the positive fraction were resuspended in RPMI 1640, supplemented with 50 IU/ml penicillin and 50μg/ml streptomycin and 10% fetal bovine serum (Invitrogen, Grand Island, USA). Cell viability, as measured by trypan blue exclusion, was more than 95%. The purity of the isolated cells as measured by flow cytometry was constantly between 90% to 95%.

Generation of DCs *in vitro*.

CD14+ monocytes were cultured in the presence of IL-4 (10ng/ml; R&D, Minneapolis, USA) and GM-CSF (50ng/ml; R&D, Minneapolis, USA) for 7 days at 37ºC in a humidified atmosphere containing 5% CO_2 as in our previous studies.[22-26] The cultures were

fed with fresh medium and cytokines on Day 3 and cell differentiation was monitored by light microscopy. For the generation of mature DC, 10μg/mL LPS (Sigma) was added for the last 2 days of the culture. On Day 7, DCs were harvested, centrifuged, washed and adjusted to 1 x 10^6 cells/mL before virus infection. The maturation of DC was confirmed by flow cytometry on a panel of maturation markers including CD40-FITC, CD80-FITC, CD83-FITC, CD86-FITC, MHC class II-FITC, CD11c-PE, MR-PC5 and CD1a–PC5 (BD PharMingen, San Diego, CA, USA).

Virus preparation, titration and infection

Laboratory procedures involving live viruses was performed in biosafety level-3 containment. SARS-CoV, strain HKU-39849 [3] was cultured in fetal rhesus kidney–4 (FRhK-4) cells. The cell culture supernatant was harvested, centrifuged to remove cell fragments, aliquoted and kept frozen at –70°C. SARS-CoV titre of the stock virus was determined by infection of FRhK-4 cells. Cytopathic changes on FRhK-4 cells was monitored every day up to 4 days and virus titre expressed as tissue culture infective dose ($TCID_{50}$). This virus titration method was also used to determine infectious virus production in SARS-CoV infected DCs.

Cells were inoculated by SARS-CoV at a multiplicity of infection (MOI) of 1. The virus was allowed to be adsorbed for 1 hour at 37°C and unbound virus was washed off by excess volume of PBS (time = 0h post infection). Mock infected cells were treated in parallel, except that virus was not added. In generating positive controls for gene expression study, Influenza A virus, H1N1 (54/98) previously isolated from human beings and prepared solely in Madin-Darby canine kidney (MDCK) cells[27] was used to infect DCs.

Transmission electron microscopy

Electron microscopy was performed on SARS-CoV infected immature and mature DCs at 0h, 6h, 12h, and 24h post infection. One million DCs were washed, fixed in 2.5% glutaraldehyde and stored at 4°C for more than 8 hours before processing. The cell suspension was post fixed in osmium tetroxide and dehydrated in a series of ethanol. After dehydration, the pellets were embedded in agar and ultra-thin sections (silver interference colour 90 nm) were cut using a diamond knife. The sections were double stained with uranyl acetate and lead citrate before being viewed on a Philips EM208S transmission electron microscope (Philips Electron Optics, Eindhoven, Netherlands) at an accelerating voltage of 80kV. More than 50 cells per section and 2-3 sections per condition/time point have been screened.

Indirect immunofluorescence assay

Mock or virus infected cells (~ 10^4 cells) harvested at 12h and 24h post infection were air dried on spotted slides and fixed with acetone:ethanol (1:1). To determine the presence of SARS-CoV and viral protein, indirect immunofluorescence assays were performed using the heat-treated convalescent serum from a known SARS patient as source of anti-SARS-CoV antibodies (SARS-CoV antibody titre of 1:640) and FITC conjugated anti-human IgG antibodies as secondary antibody (INOVA Diagnostics Inc., San Diego, CA, USA). Evan's Blue was used as the counterstain. Fluorescent images were acquired using a Leica DMLB microscope and a Leica DC500 digital camera system with the Leica Image Manager software (Leica Microsystems AG, Wetzlar, Germany). Confocal microscopy was performed by Bio-Rad Radiance 2100 laser scanning confocal system equipped with Nikon E1000 microscope and the LaserSharp2000 software (Bio-Rad Laboratories Inc., CA, USA).

Quantification of SARS-CoV RNA by real-time quantitative PCR

Total RNA was extracted from ~5 x 10^5 cells harvested at 3h, 9h, 24h, Day 3 and Day 6 post infection by the RNeasy Mini Kit (Qiagen, Hilden, Germany). Two microlitres of DNase-treated total RNA was reverse transcribed by either forward or reverse primers specific for SARS-CoV (Table 1) and Superscript II reverse transcriptase (Invitrogen Life Technologies, USA) according to the manufacturer's recommendation. The forward primers transcribe the negative strands whereas the reverse primers transcribe the positive strands into cDNA. The cDNA was diluted (1:20) and quantified by real-time PCR using the Lightcycler Technology (Roche, Mannheim, Germany) as in our previous study.[4] Detection of PCR product was based on SYBR green fluorescence signal. The standard curve was generated using serial dilutions of plasmids (~10 – 10^{10} copies) containing cloned sequences involved.

Active caspase-3 assay

Activated caspase-3 was selected as a biological marker for apoptosis. Mock or SARS-CoV infected adult immature DCs were assayed using a monoclonal active caspase-3 antibody apoptosis kit according to manufacturer's specification (BD PharMingen, San Diego, CA, USA). Briefly, cultured cells harvested at different time points were washed twice with PBS, then fixed and permeabilised in a solution containing pH-buffered saline, saponin and 4% (w/v) paraformaldehyde for 20 min on ice. Cells were then washed twice with a buffer containing fetal bovine serum, sodium azide and saponin, and stained by monoclonal antibody against active caspase-3. Stained cells were washed, resuspended and analysed by flow cytometry (COULTER EPICS ELITE, Beckman Coulter Corporation, Miami, Florida, USA). Ten thousand events per sample were collected into listmode files and analyzed by the WINMDI 2.8 analysis software. For positive control, Jurkat T cells (ATCC TIB-152) were induced to undergo apoptosis by 25ng/mL of anti-Fas antibodies (clone CH11,

Upstate USA Inc. NY, USA) for 3 or 24h, harvested and stained in parallel with the mock or SARS-CoV infected DCs.

Determination of surface marker expression on DCs by flow cytometry

Mock or SARS-CoV infected adult immature DCs (MOI = 1) were harvested at 48h post infection, fixed in 4% paraformaldehyde and stored at 4°C for more than 1 hour before analysis. The fixed cells were washed and stained for a panel of maturation markers (including CD83-FITC, CD86-FITC, MHC class I-FITC and MHC class II-FITC) and isotype control-FITC (BD PharMingen, San Diego, CA, USA). To determine if SARS-CoV infection will impair DC maturation, LPS (10μg/mL) was added to the SARS-CoV infected immature DCs throughout the post infection period. Mock infected cells were included as control of the experiment.

Quantification of cytokine/chemokine RNA by real-time quantitative PCR

Cells (~1.5 x 10^5) were harvested at 3h and 9h post infection and total RNA was extracted by TRIzol Reagent (Invitrogen Life Technologies, USA). In later experiments, QiaShredder columns (Qiagen, Hilden, Germany) were used to ensure adequate homogenisation and RNA was extracted by the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed on the DNase-treated total RNA using oligo (dT) primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, USA) according to the manufacturer's recommendation. The cDNA synthesised were diluted (1:50) and quantified by real-time PCR using the Lightcycler Technology (Roche, Mannheim, Germany) or Taqman Technology (Applied Biosystems, CA, USA). Specific primers (Table 1) were used and non specific reactions and primer-dimer artifacts have been minimised (as evaluated by gel electrophoresis). Detection of PCR product was based on SYBR green or

Taqman fluorescence signal. Dissociation curve analysis was performed after SYBR green assays to ensure specific target detection. The β actin gene was amplified as an internal control. Standard curves were generated using serial dilutions of plasmids (~10 – 10^{10} copies) containing cloned sequences involved. Results were calculated as the number of targeted molecules/μL cDNA. To standardise results for variability in RNA and cDNA quantity and quality, we express the results as the number of target copies per 10^4 copies of β actin gene.[27]

Statistical analysis

All data were expressed as mean±SEM. All samples were paired and differences between groups analyzed by paired Student t test or the non-parametric equivalents.

Results

SARS-CoV could infect both immature and mature DCs from adult and CB

In the initial experiments, we determined if SARS-CoV can infect DCs by electron microscopy and immunofluorescence staining. Since the route of entry of SARS-CoV into DCs have not been identified, we used both immature and LPS treated mature DCs which have different expression of receptors and different endocytotic functions. We also used DCs derived from adult and cord blood monocytes to determine if the developmental status of the host would affect virus entry.

Electron microscopy showed SARS-CoV (black arrows) binding to DC (Fig. 1a) and adsorbed in either an invagination of the plasma membrane or an endosome (Fig. 1b) at 0h post infection. At 6h, 12h and 24h post infection, viral particles were detected in endosomes (Fig. 1c) and cytoplasm (Fig.1d) but not in the Golgi apparatus (Fig. 1e). No virus budding (Fig. 1f) was observed in all the cells examined (n>200). Cytopathic effect was observed in some immature DCs but to a lesser extent in the mature DCs (data not shown). Similar finding was observed in adult and cord blood immature and mature DCs from 3 independent donors.

Using convalescent serum from SARS patients as source of anti-SARS-CoV antibodies, positive immunofluorescence staining was detected in SARS-infected DCs but not the mock infected DCs (Fig. 2). The observations in adult and CB DCs were similar and a representative case of CB DCs is included. Similar staining was detected in both immature and mature monocyte derived DCs with over 90% of DCs being positive at both 12h (Fig. 2b) and 24h post infection (Fig. 2c). Confocal microscopy showed that positive staining was not limited to the cell surface but inside cytoplasm (Fig. 2d).

Incomplete replication of SARS-CoV was detected in adult immature DCs

SARS-CoV is a positive single stranded RNA virus and the detection of negative stranded RNA (the negative RNA template) may be an indication of viral replication.[28] Using specific forward and reverse primers for SARS-CoV, both negative and positive strands of SARS-CoV were detected in infected DCs (Fig. 3) but not in the mock infected cells (data not shown; n=3). The pattern of expression for the negative and positive strands were similar. There was a rapid decline in viral RNA from 3h to 9h post infection but due to sample variation, the difference did not yield statistical significance. No change in virus RNA expression was detected in later time points of 24h, Day 3 and Day 6.

We determined the presence of infectious SARS-CoV in adult immature DCs and their culture supernatant by half log titration cytopathic assay using FRhK-4 cells (n = 3). SARS-CoV infected DCs harvested on Day 1 - 6 post infection were washed and resuspended in 500 μL of PBS. The cells were disrupted by freezing and thawing once at -70°C. The virus titre from the cell pellet decreased from 2 to <1 log of $TCID_{50}$/4 x 10^5 cells from Day 1 to Day 6. In cell culture supernatant, the virus titre on Day 1 ranged from <1 to 2 log of $TCID_{50}$/100μL. No increase in virus titre was detected in cell culture supernatant from Day 1 to Day 6.

SARS-CoV did not induce apoptosis nor maturation in adult immature DCs

Similar percentages of active caspase-3 positive cells was observed in mock and SARS-CoV infected adult immature DCs at 6h, 12h and 24h post infection (n = 4; $p>0.05$; Fig. 4). In the positive control, the percentage of active caspase-3 positive Jurkat cells were 15% and 35% at 3h and 24h post addition of anti-Fas antibodies respectively.

As shown by flow cytometric analysis (Figure 5), SARS-CoV alone did not upregulate the expression of CD83, CD86, MHC Class I and MHC Class II on adult immature DCs.

However SARS-CoV infected cells can be stimulated by LPS (10μg/mL) to upregulate the expression of these molecules to similar levels as in the mock infected controls.

SARS-CoV did not stimulate the gene expression of interferons or IL-12 in immature DCs

Based on our previous observation of induced proinflammatory cytokines in human macrophages by the avian influenza virus H5N1,[27] we quantitated the mRNA expression of representative cytokines and chemokines in SARS-CoV infected adult immature DCs. Low level of IFN-α, IFN-β, IFN-γ and IL-12p40 expression (average in range of 0 - 30 copies per 10^4 β-actin) was observed in SARS-CoV infected DCs (Fig. 6). The IFN-β and IL-12p40 mRNA levels were marginally elevated from 3h to 9h post infection but the differences did not reach statistical significance. The level of IFN-γ was significantly higher in SARS-CoV infected CB DCs than adult DCs (Table 2).

SARS-CoV stimulated moderate expression of proinflammatory genes in immature DCs

The production of proinflammatory cytokines TNF-α and IL-6 were in the range of 0 - 400 copies per 10^4 β-actin. Comparing the mock and SARS-CoV infected DCs, there was a moderate induction of TNF-α and IL-6 at both 3h and 9h post infection (Fig. 7). The upregulation of IL-6 in SARS-CoV infected CB DCs was significantly higher than that in adult DCs (Table 2).

SARS-CoV upregulated chemokines gene expression in immature DCs

The gene expression of inflammatory chemokines, MIP-1α, RANTES, IP-10 and MCP-1, were all significantly upregulated in SARS-CoV infected DCs (Fig. 8). The induction of gene expression in DCs by SARS-CoV was strongest for IP-10 and MCP-1. The

expressions of MIP-1α, IP-10 and MCP-1 genes in both mock and SARS-CoV infected CB DCs were significantly higher than that in adult DCs (Table 2). Similar finding was observed for RANTES but due to sample variations the difference betweeen CB and adult DCs was not statistically significant.

Discussion

Severe acute respiratory syndrome is a recently described infectious disease that the human population has no prior immune experience.[29] Therefore, the SARS outbreak in 2003 provides an unique opportunity for the study of human response to a novel virus. The clinical presentation of SARS patients suggested that SARS-CoV might have specific mechanisms to escape from normal immune responses. In this study, we focused on DCs, which are the key antigen presenting cells that play crucial roles in the anti-viral immune response including the priming of specific T cell response.

The binding and entry of SARS-CoV was shown by electron microscopy (Fig. 1) and further confirmed by immunofluorescence staining (Fig. 2). Similar to other viruses, the uptake of SARS-CoV into DCs may be through macropinocytosis, receptor binding leading to endocytosis or membrane fusion. Macropinocytosis is a non-specific mechanism for virus internalisation.[30] In previous study, we have demonstrated that mature DCs have lower endocytotic function than immature DCs.[22] Our observation of similar infectivity of SARS-CoV in both immature and mature DCs from adult or CB suggested that SARS-CoV entry to DCs is not dependent on the efficiency of endocytosis.

Aminopeptidase N (CD13), which is the receptor responsible for the entry of another human CoV (229E) into intestinal, lung and kidney epithelial cells[31] is present on DCs.[32,33] Despite initial speculation that CD13 may be involved in SARS, there is no evidence to support its role. Angiotensin-converting enzyme (ACE)-2 has now been identified as a functional receptor for SARS-CoV[34] and the tissue distribution of ACE-2 has been studied extensively.[35,36] In line with the report on the lack of ACE2 protein in immune cells,[35] we did not detect any gene expression of ACE-2 in purified monocytes nor DCs (data not shown).

Our results suggested that cell types lacking ACE-2 may also be infected by SARS-CoV and other receptors may be involved in virus entry.

Initial postgenomic characterization of the SARS-CoV has revealed 23 potential N-linked glycosylation sites,[37] and some of the sites of the surface spike (S) protein are of high-mannose structure.[38] Hence, the uptake of SARS-CoV into DCs may also be mediated through binding to c-type lectin receptors, such as mannose receptor (CD206), DC-SIGN (CD209), langerin (CD207) and DEC-205 (CD205).[9] This hypothesis is supported by a recent report that retroviral vectors pseudotyped with the SARS-CoV S protein can bind to DC-SIGN and enter mature DCs.[39] Cell mediated transfer of the pseudovirus to Vero cells was also demonstrated, further supporting an important role played by DCs.

Viral infection usually results in a full replication cycle with production of progeny viruses. However, human CoV is known to be difficult to culture *in vitro* and only FRhK-4[3] and Vero E6 cells[40] were reported to be permissive for SARS-CoV replication. In this study, the replication of SARS-CoV in DCs appeared to be incomplete – there was expression of the viral genome (including the negative and positive RNA templates, Fig. 3) but there was no increase in viral copies over 6 days of culture, no virus budding nor production of infectious virus into the culture medium.

Human CoV (229E) has been shown to induce apoptosis in monocytes/macrophages[41] and some viruses, such as measles,[42] also induce apoptosis of DCs. In this study, we did not observe significant cell death in SARS-CoV infected DCs under light and electron microscopy nor in active caspase-3 assays (Fig. 4). This result suggested that the immunosuppressive effect of SARS-CoV may not be mediated through direct cytopathic effect on DCs.

Some viral infections, such as influenza,[43] promote DC maturation which results in enhanced killing of the virus by the host. However, some viruses, such as measles, herpes

simplex, dengue viruses, suppress DC maturation by inhibiting the expression of co-stimulatory and MHC molecules.[12] For example, vaccinia virus inhibits the expression of CD83, CD86 and MHC Class II molecules in immature DCs. [44] The decrease in CD86 and MHC Class II molecules may lead to antigenic tolerance and decreased antigen presentation respectively. Although our results demonstrated that SARS-CoV did not upregulate the expression of CD83, CD86, MHC Class I nor MHC Class II molecules on immature DCs, maturation of SARS-CoV infected DCs can still be induced by LPS suggesting SARS-CoV did not impair DC maturation (Fig. 5).

Viruses enhance their own survival by interfering with normal innate immune responses of the host. Usually, Type 1 interferons are effectively generated in response to viral infection and keep activated T cells alive.[45] However, very low level of interferon gene expression was observed in SARS-CoV infected DCs (Fig. 6). In general, double stranded RNA, when bound to toll-like receptor 3, triggers the production of interferons in DCs.[46] The lack of activation of interferon production by SARS-CoV may involve mechanisms that interferes with downstream signaling molecules such as IFN regulatory factor–3 (IRF-3), TRIF (TIR domain-containing adapter inducing IFN), and putative kinases or proteases.[47] Since DCs and interferons both play a role in the generation of antibody responses,[48] the lack of interferon induction in DCs may contribute to the slow antibody production and progressive increase of viral load over the first 10 days of SARS observed clinically.[4] Interestingly, SARS-CoV infected CB DCs expressed slightly higher levels of interferon genes than adult DCs. Further investigation is needed to determine if this observation is relevant to the less severe disease presentation observed in pediatric SARS.

Interleukin 12 is the main cytokine secreted by DCs that regulates the differentiation of CD4+ T cells into Th1 cells and serves important function in cell-mediated immunity.[49] Similar to the observation in measles virus infected DCs,[42] the lack of IL-12 production in

SARS-CoV infected DCs might suppress Th1 and favor Th2 responses. Furthermore, the immune escape mechanism operated in HIV[14,15] and dengue virus[50] via DC-SIGN leading to suppressed IL-12 production may also be implicated in SARS.

Both TNF-α and IL-6 are proinflammatory cytokines which regulate apoptosis, cell proliferation, differentiation, immunity, and inflammation.[51] We have previously reported that the severity of avian flu may be due to high TNF-α production in macrophages infected by H5N1 viruses.[27] Likewise, SARS patients were treated with corticosteroids in the belief that local production of proinflammatory cytokines is responsible for the immunopathology in the lungs.[52] However, we only detected a slight upregulation of TNF-α and IL-6 mRNA expressions in SARS-CoV infected DCs (Fig. 7).

Chemokines are chemotactic messengers that play important roles in leukocyte recruitment.[53] Upregulation of chemokines has also led to chemokine-mediated host pathology in viral diseases.[54] In concordance with the detection of high plasma concentration of chemokines in SARS patients,[55] Glass and coworkers reported that there is massive upregulation of chemokines in the lungs of SARS-CoV infected mice.[56] In this study, we also observed significant upregulation of representative inflammatory chemokines in SARS-CoV infected DCs (Fig. 8). MIP-1α, RANTES, IP-10 and MCP-1 are CXC chemokines without the ELR (glutamic acid-leucine-arginine)-motif. They preferentially act on mononuclear cells and their upregulation has been detected in influenza A virus infection.[57] These chemokines may be responsible for the recruitment and adhesion of inflammatory cells, and the migration of leukocytes into the tissue.[57] Our findings will need to be substantiated by *in vivo* experiments.

More importantly, chemokines are also implicated in the autocrine regulation of DC migration to draining lymph nodes.[19,20,58] We hypothesize that migrating virus-infected DCs may facilitate the virus spread, skew T cell responses through altered cytokine production or

induce apoptosis in T cells leading to immunosuppression.[59] Interestingly, we detected significantly higher level of chemokine genes (20 – 100 folds) in CB DCs than in adults DCs, the relevance of which requires further study.

Overall, we concluded that the lack of antiviral cytokine response against a background of intense chemokine upregulation could represent a mechanism of immune evasion by SARS-CoV.

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Table 1 PCR primers and probes

Genes	Sequences	Sizes (bp)
SARS-CoV	(F) 5′ TAC ACA CCT CAG CGT TG 3′	182
	(R) 5′ CAC GAA CGT GAC GAA T 3′	
β-actin	(F) 5′ CCC AAG GCC AAC CGC GAG AAG AT 3′	219
	(R) 5′ GTC CCG GCC AGC CAG GTC CAG 3′	
IFN-α	(F) 5′ CCT TCC TCC TGT CTG ATG GA 3′	67
	(P) 5′ (FAM) CAG ACA TGA CTT TGG ATT TCC CCA GG (TARMA) 3′	
	(R) 5′ ACT GGT TGC CAT CAA ACT CC 3′	
IFN-β	(F) 5′ GCC GCA TTG ACC ATC T 3′	261
	(R) 5' AGG AGT ACA GTC ACT GTG 3′	
IFN-γ	(F) 5′ CTA ATT ATT CGG TAA CTG ACT TGA3′	75
	(P) 5′ (FAM) TCC AAC GCA AAG CAA TAC ATG AAC (TARMA) 3′	
	(R) 5′ ACA GTT CAG CCA TCA CTT GGA 3′	
TNF-α	(F) 5′ GGC TCC AGG CGG TGC TTG TTC 3′	409
	(R) 5′ AGA CGG CGA TGC GGC TGA TG 3′	
IL-6	(F) 5′ ATT CGG TAC ATC CTC GAC 3′	331
	(R) 5′ GGG GTG GTT ATT GCA TC 3′	
IL-12	(F) 5′ CGG TCA TCT GCC GCA AA 3′	80
	(R) 5′ TGC CCA TTC GCT CCA AGA 3′	
MIP-1α	(F) 5′ CTC TGC ACC ATG GCT CTC TGC AAC 3′	98
	(R) 5′ TGT GGA ATC TGC CGG GAG GTG TAG 3′	
RANTES	(F) 5′ CCC CTC ACT ATC CTA CC 3′	285
	(R) 5′ TCA CGC CAT TCT CCT G 3′	
IP-10	(F) 5′ CTG ACT CTA AGT GGC ATT 3′	208
	(R) 5′ TGA TGG CCT TCG ATT CTG 3′	
MCP-1	(F) 5′ CAT TGT GGC CAA GGA GAT CTG 3′	91
	(R) 5′ CTT CGG AGT TTG GGT TTG CTT 3′	
ACE2	(F) 5′ CTG GGA TCA GAG ATC GGA AGA 3′	288
	(R) 5′ CTC TCT CCT TGG CCA TGT TGT 3′	

F, forward primers; R, reverse primers; P, Taqman probes
TNF, tumor necosis factor; IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; IP-10, Interferon-γ induced protein 10; MCP-1, Monocyte chemoattractant protein 1, ACE2, angiotensin converting enzyme 2

Table 2 **CB immature DCs expressed significantly higher level of some cytokines and chemokine genes than adult immature DCs****

	3h p.i.		9h p.i.	
	MOCK	**SARS-CoV**	**MOCK**	**SARS-CoV**
IFN-α	-	-	-	-
IFN-β	-	-	-	-
IFN-γ	-	**p = 0.03**	-	-
IL-12p40	**p = 0.02**	-	-	-
TNF-α	-	-	-	-
IL-6	**p = 0.04**	-	-	**p = 0.006**
MIP-1α	**p = 0.03**	**p = 0.003**	**p = 0.01**	**p = 0.003**
RANTES	-	-	-	-
IP-10	**p = 0.03**	-	-	**p = 0.03**
MCP-1	**p = 0.03**	**p = 0.005**	**p = 0.01**	**p = 0.02**

** Adult DCs (n = 7) CB DCs (n = 5)

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Figure Legends

Figure 1 Electron microscopy of SARS-CoV infected human DCs

Negative-contrast thin section transmission electron micrograph of SARS-CoV infected human DCs showed virus (black arrows) binding (a) and uptake (b) at 0h post infection. At 6h, 12h and 24h post infection, virus particles were detected in endosomes (c) and cytoplasm (d) but not in the Golgi apparatus (e). No virus budding was observed (f) in all cells examined. Images are representative of SARS-CoV infected human DCs from 3 independent adult or CB donors.

Figure 2 Immunofluorescence assay for SARS-CoV detection in human DCs

Positive immunofluorescence staining was detected in human immature and mature DCs at 12h (b) and 24h (c) after infection with SARS-CoV (MOI = 1). Confocal microscopy showed positive staining in the cytoplasm of DCs (d). Images are representative of immature and mature DCs from 11 independent adult or CB donors.

Figure 3 Viral gene expression in SARS-CoV infected human adult immature DCs by quantitative RT-PCR

Both negative (a) and positive strands (b) of SARS-CoV Replicase 1b mRNA were detected in SASR-CoV infected adult immature DCs at 3h, 9h, 24h, Day 3 and Day 6 post infection (MOI = 1). Similar pattern of decreased viral gene expression was detected for the negative and positive strands in all three cases.

Figure 4 Active caspase 3 assay for apoptosis in human adult immature DCs

Comparing to mock infected adult immature DCs, no significant induction of active caspase 3 positive cells was observed at 6h, 12h and 24h post infection ($n = 4$; $p>0.05$). Data are shown as mean ± SEM of DCs from 4 independent donors. The percentage of active caspase-3 positive Jurkat cells in the positive control at 3h and 24h post addition of anti-Fas antibodies were 15% and 35% respectively.

Figure 5 Flow cytometry analysis of cell surface molecules expression on human adult DCs

Mock or SARS-CoV infected adult immature DCs (MOI = 1) were harvested at 48h post infection and stained for flow cytometry analysis. Surface staining is shown by filled histogram and isotype control is marked by the dotted line. SARS-CoV alone did not upregulate the expression of CD83, CD86, MHC Class I and MHC Class II. However SARS-CoV infected cells can be stimulated by LPS (10μg/mL; think line) to upregulate the expression of these molecules to similar levels as in the mock infected controls. Data shown is representative of adult immature DCs from 5 independent donors.

Figure 6 Antiviral cytokine gene expression profile of SARS-CoV infected human immature DCs by quantitative RT-PCR

Antiviral cytokine mRNA concentrations in adult (a) and CB (b) immature cells were assayed at 3h and 9h after infection with SARS-CoV (MOI = 1). Mock infected cells were included as negative control. The concentrations were normalised to those of β-actin mRNA in the corresponding sample. There were low expressions of IFN-α, IFN-β, IFN-γ and IL-12p40 genes in SARS-CoV infected DCs. Data are shown as mean ± SEM (adult $n = 7$; CB $n = 5$)

Figure 7 Proinflammatory cytokine gene expression profile of SARS-CoV infected human immature DCs by quantitative RT-PCR

Proinflammatory cytokine mRNA concentrations in adult (a) and CB (b) immature cells were assayed at 3h and 9h after infection with SARS-CoV (MOI = 1). Mock infected cells were included as negative control. The concentrations were normalised to those of β-actin mRNA in the corresponding sample. There were moderate upregulation of TNF-α and IL-6 expression in SARS-CoV infected DCs. Data are shown as mean ± SEM (adult n = 7; CB n = 5; * $p < 0.05$)

Figure 8 Chemokine gene expression profile of SARS-CoV infected human immature DCs by quantitative RT-PCR

Chemokine mRNA concentrations in adult (a) and CB (b) immature cells were assayed at 3h and 9h after infection with SARS-CoV (MOI = 1). Mock infected cells were included as negative control. The concentrations were normalised to those of β-actin mRNA in the corresponding sample. There were significant upregulation of MIP-1α, RANTES, IP-10 and MCP-1 in SARS-CoV infected DCs. Data are shown as mean ± SEM (adult n = 7; CB n = 5 * $p < 0.05$)

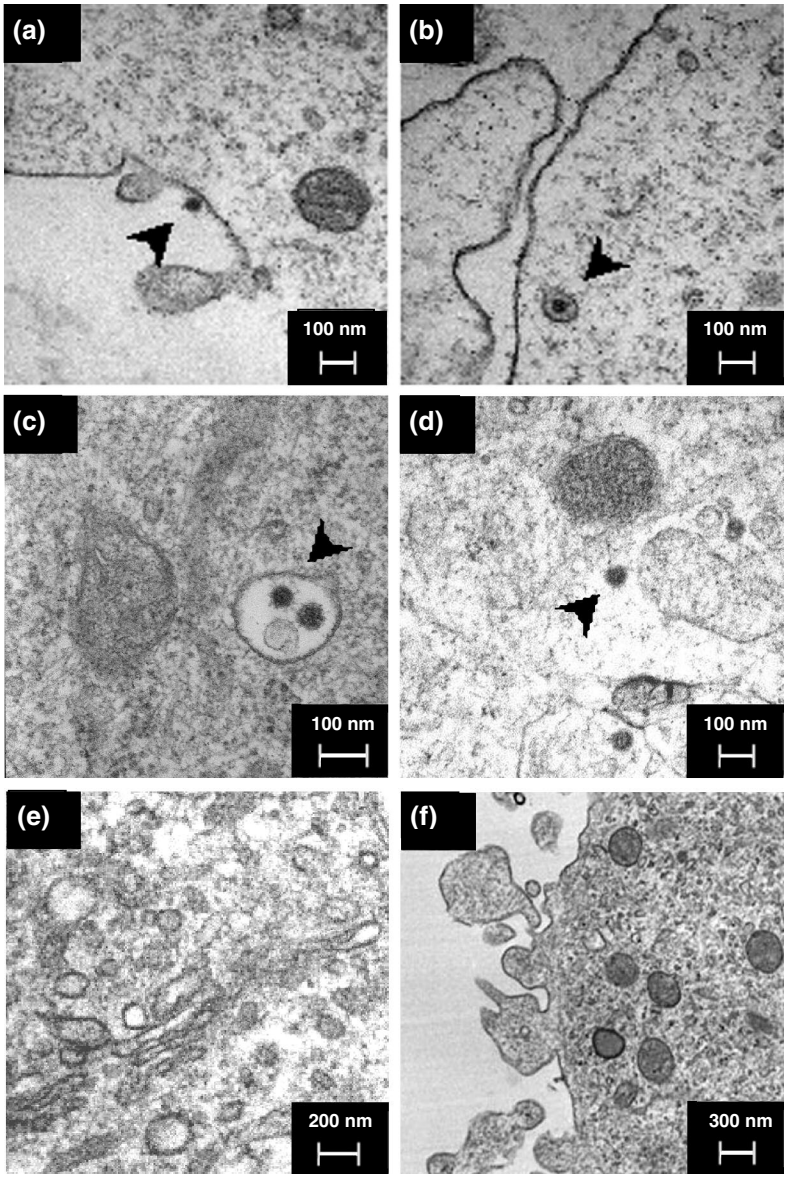


Figure 1

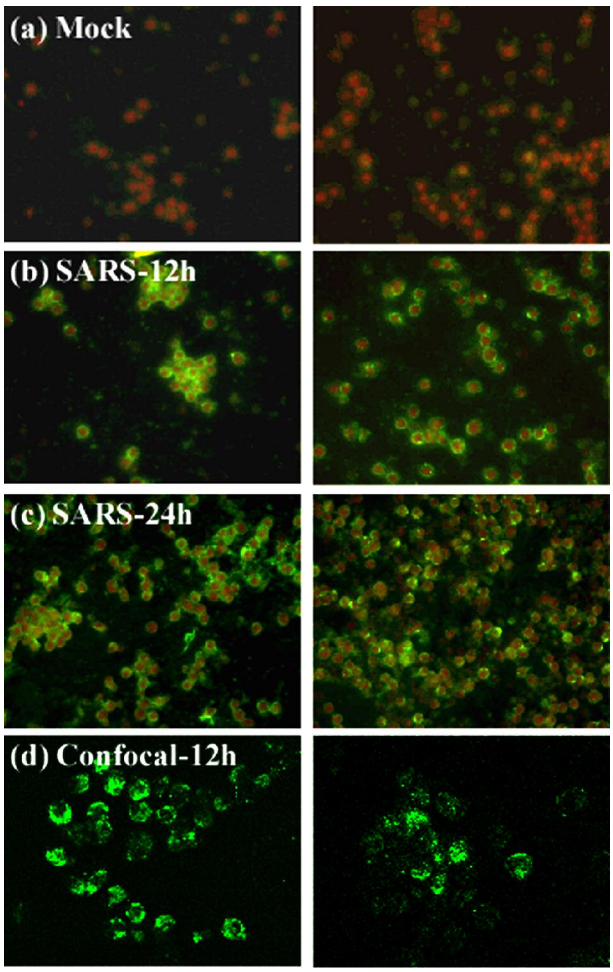
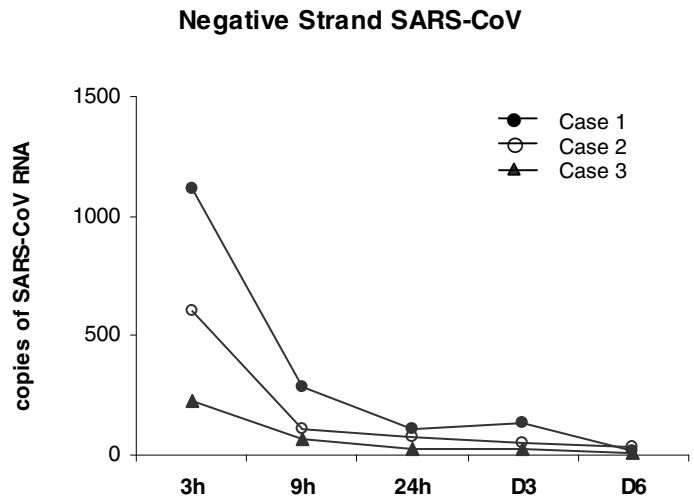

(a) Mock
(b) SARS-12h
(c) SARS-24h
(d) Confocal-12h
immature DCs
LPS-treated
mature DCs

Figure 2

(a)


Negative Strand SARS-CoV
copies of SARS-CoV RNA
1500
1000
500
0
3h
9h
24h
D3
D6
Case 1
Case 2
Case 3

(b)

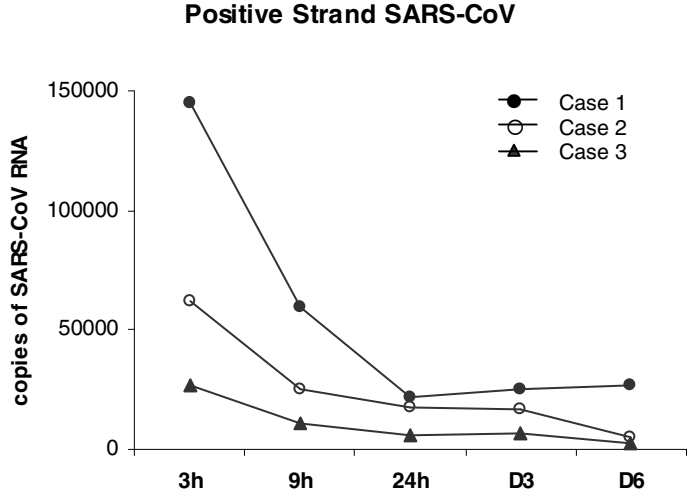

Positive Strand SARS-CoV
copies of SARS-CoV RNA
150000
100000
50000
0
3h
9h
24h
D3
D6
Case 1
Case 2
Case 3

Figure 3

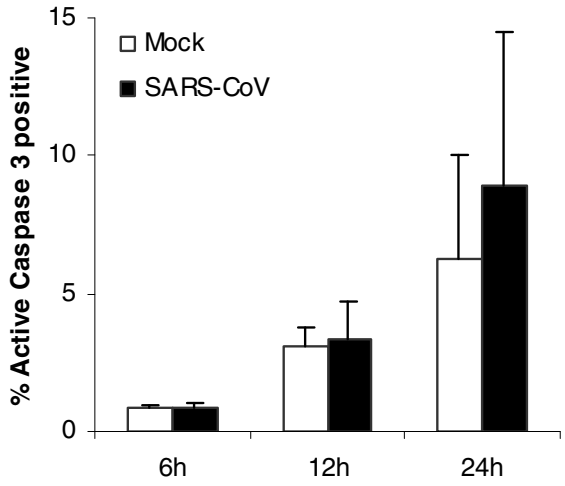


Figure 4

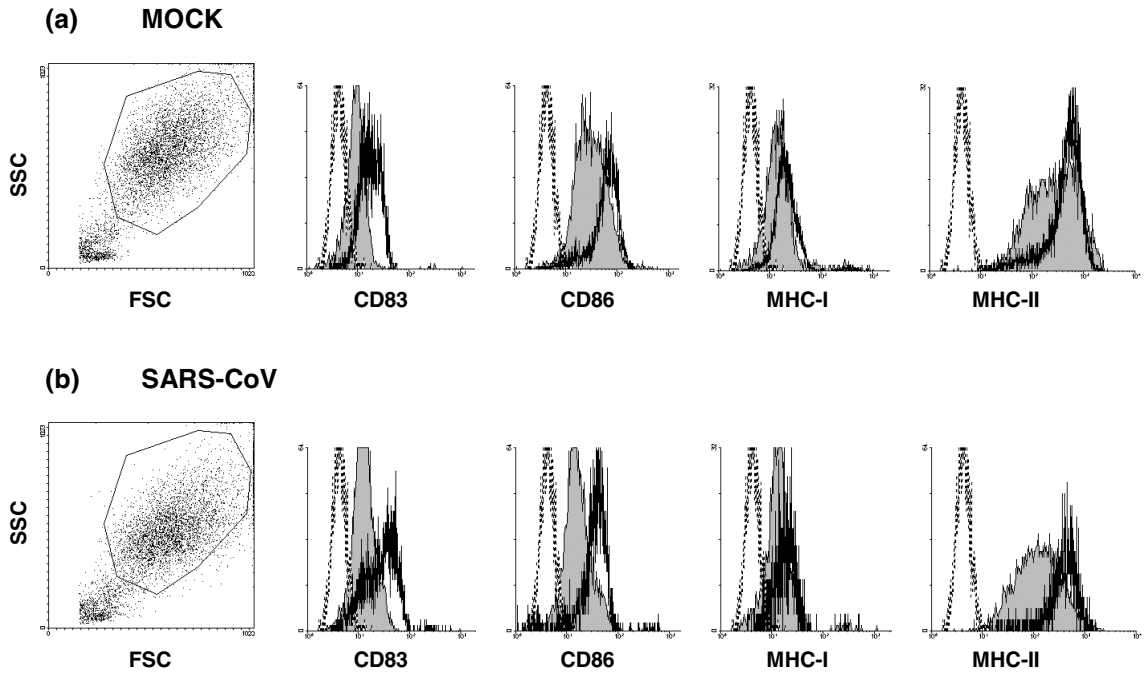

(a) MOCK
SSC
FSC
CD83
CD86
MHC-I
MHC-II
(b) SARS-CoV
SSC
FSC
CD83
CD86
MHC-I
MHC-II

Figure 5

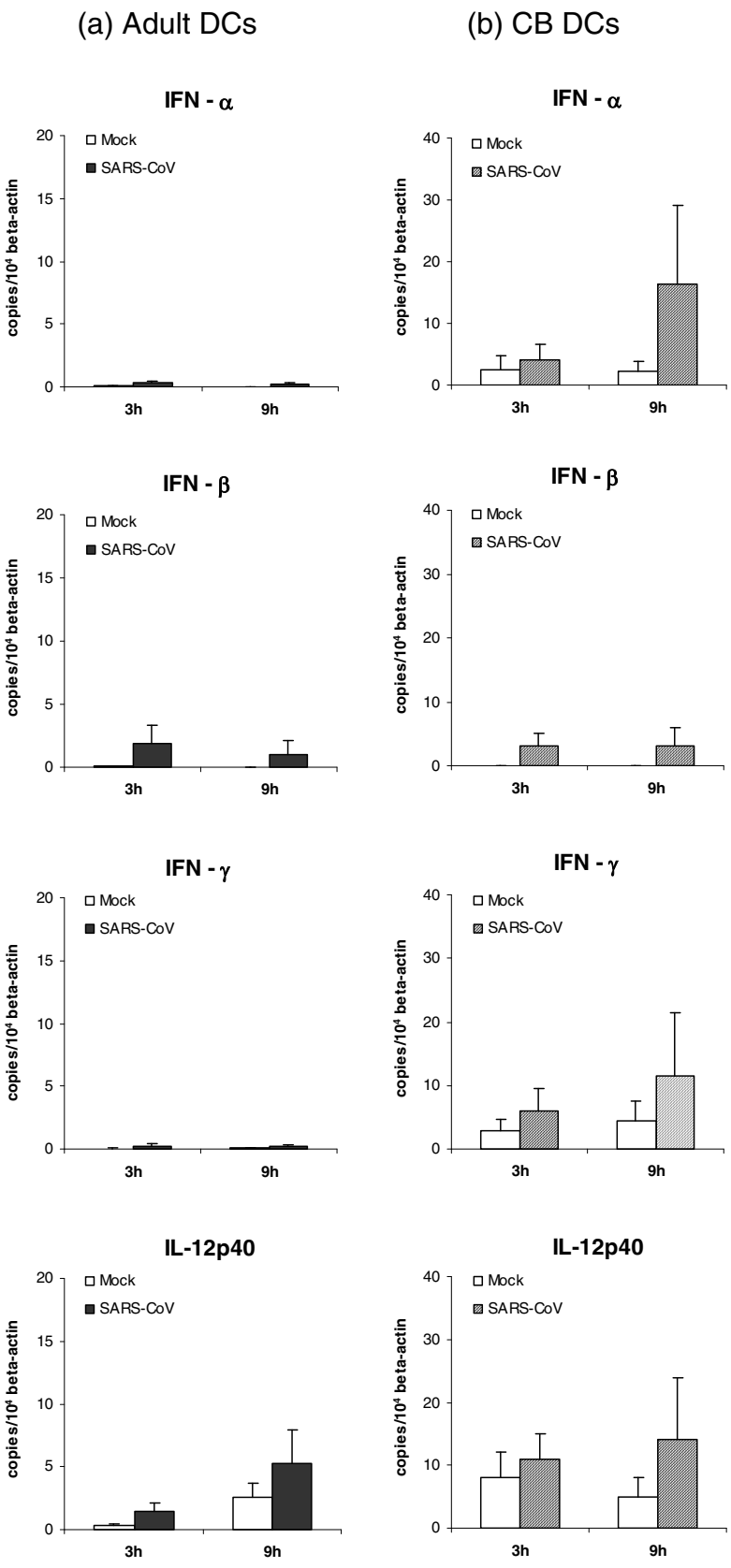

(a) Adult DCs
(b) CB DCs
IFN - α
IFN - β
IFN - γ
IL-12p40
Mock
SARS-CoV
copies/10⁴ beta-actin
3h
9h

Figure 6

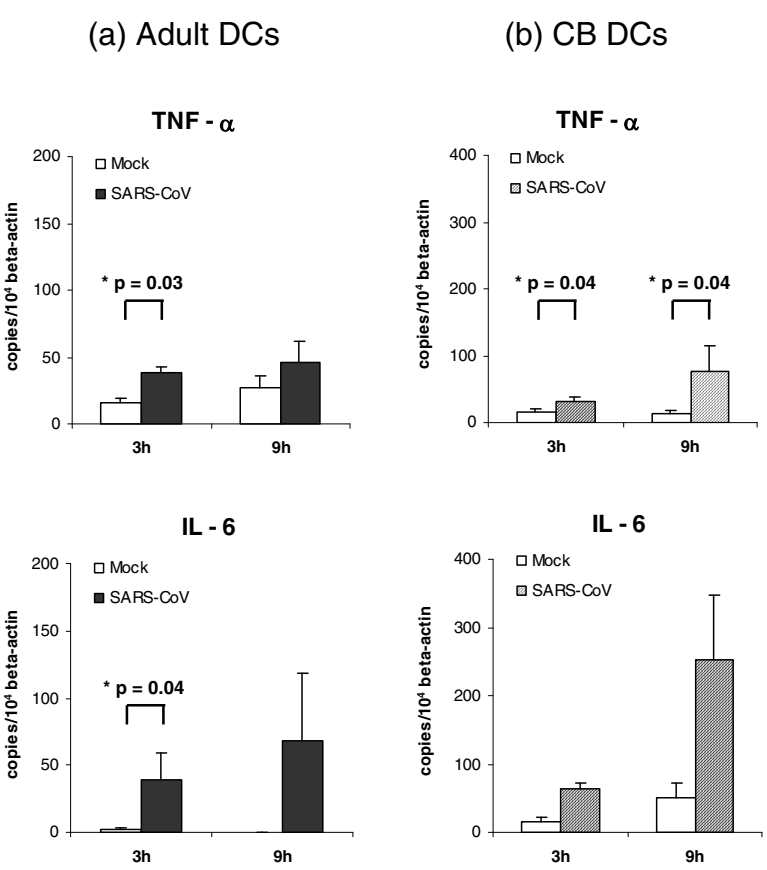

(a) Adult DCs
(b) CB DCs
TNF - α
IL - 6
Mock
SARS-CoV
copies/10⁴ beta-actin
* p = 0.03
* p = 0.04
* p = 0.04
* p = 0.04
3h
9h
0
50
100
150
200
300
400

Figure 7

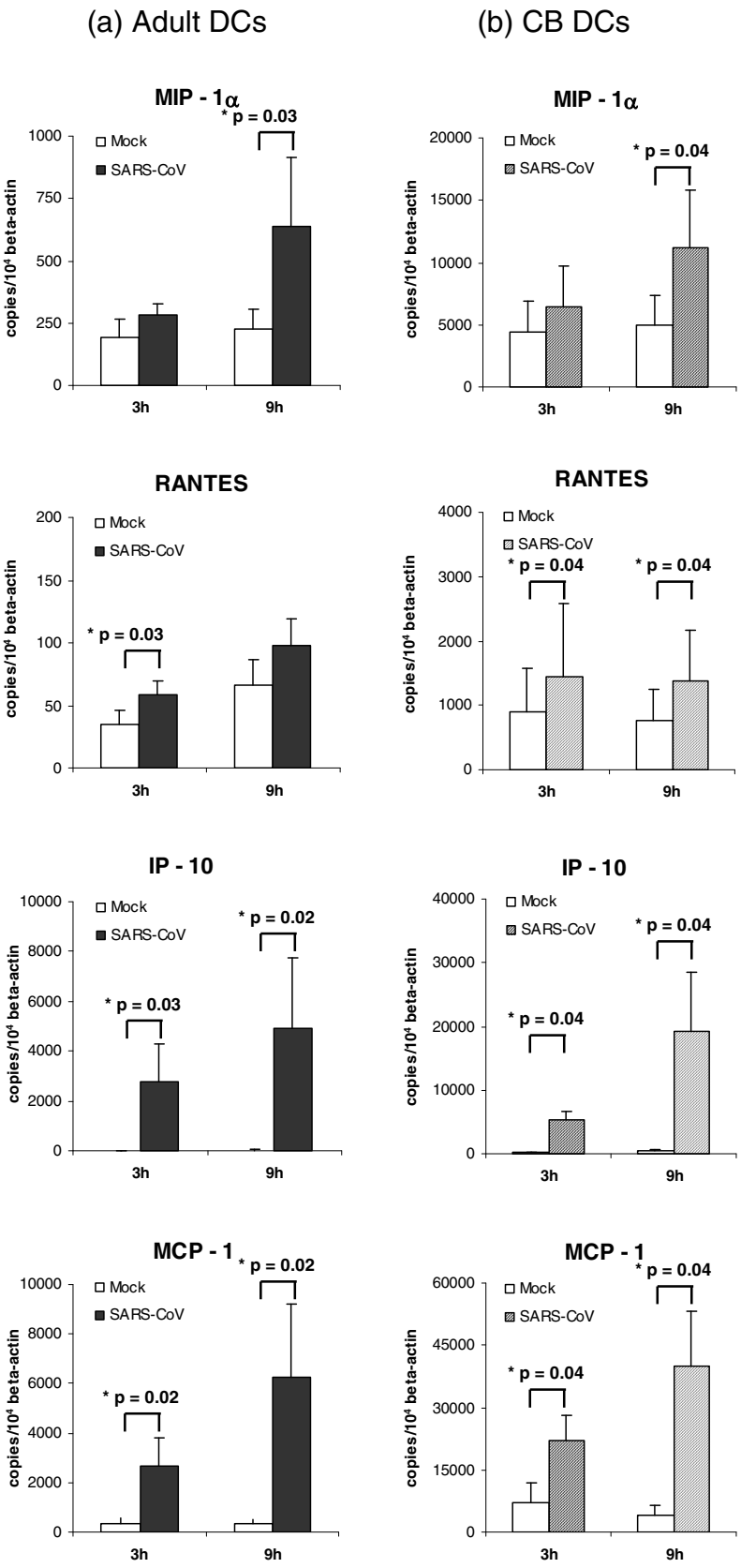

(a) Adult DCs
(b) CB DCs
MIP - 1α
RANTES
IP - 10
MCP - 1
Mock
SARS-CoV
copies/10⁴ beta-actin
3h
9h
* p = 0.03
* p = 0.04
* p = 0.02

Figure 8