Virology 390 (2009) 157–162

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Rapid Communication

Crimean Congo hemorrhagic fever virus infects human monocyte-derived dendritic cells

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ARTICLE INFO

Article history:
Received 27 March 2009
Returned to author for revision 20 April 2009
Accepted 5 June 2009

Keywords:
Crimean Congo hemorrhagic fever virus
Cytokines
Innate immunity
Dendritic cells
CCHFv

ABSTRACT

For some patients infection with Crimean Congo hemorrhagic fever virus (CCHFV) causes a severe disease characterized by fever, vascular leakage and coagulopathy. Knowledge of CCHF pathogenesis is limited and today there is no information about the specific target cells of CCHFV. In this study we analyzed the permissiveness of human peripheral blood mononuclear cells (PBMCs) including monocyte-derived dendritic cells (moDCs) to CCHFV infection. Interestingly, we found that moDCs are the most permissive to CCHFV infection and this infection induced cytokine release from moDCs. Furthermore, supernatants from infected moDCs were found to activate human endothelial cells.

Introduction

Crimean Congo hemorrhagic fever virus (CCHFV) belongs to the *Nairovirus* genus in the *Bunyaviridae* family. It can cause a severe human disease termed Crimean Congo hemorrhagic fever (CCHF), with mortality rates ranging from 5 to 70% (Bakir et al., 2005; Baskerville et al., 1981; Schwarz et al., 1997). CCHFV is endemic in large parts of the world and considered a threat to public health in these regions. Second after Dengue, it is the most widespread of the medically important arthropod-borne viruses. Virus transmission is known to occur by tick bites from *Hyalomma* ticks, and by contact with infected animal blood or tissues. Person to person transmission can occur via body fluids or through aerosol from patients in advanced stages of disease (Swanepoel et al., 1987). Handling of the virus requires specialized biosafety level 4 laboratories (BSL-4), a restriction resulting in limited knowledge regarding the pathogenesis of CCHF.

Unlike other BSL-4 pathogens, there currently is no animal model for studying sequential events leading to the hemorrhagic fever resulting from CCHFV infection; therefore target cell determination must occur *in vitro*. In order to address this issue we sought to determine the target cell populations for CCHFV infection by infecting human peripheral blood mononuclear cells (PBMCs), B cells, T cells, NK cells, monocytes and monocyte-derived dendritic cells (moDCs), and analyzing their response to infection by measuring cytokine release. The biological activity of supernatants derived from moDCs was further analyzed by exposing human umbilical vein endothelial cells (HUVECs) to these supernatants and quantifying intercellular adhesion 1 (ICAM-1) mRNA expression.

Results

Monocyte-derived dendritic cells are productively infected by CCHFV

PBMCs were purified from buffy coats and subsequently sorted by fluorescence activated cell sorting (FACS) into cell subsets consisting of B cells, T cells, NK cell and monocytes. MoDCs were also generated following 6-day culture with IL-4 and GM-CSF. The purity of all the sorted cell populations was consistently >95% as shown in Fig. 1A. The sorted cells were infected with CCHFV at a MOI of 10 and RNA extracted 24 and 48 hs post infection (hpi) to quantify the CCHFV RNA levels in the infected versus uninfected cells. In contrast to other cells, CCHFV RNA was found in moDCs at 24 hpi and more pronounced levels were measured at 48 hpi (Fig. 1B). Further verification of moDC susceptibility to CCHFV infection was performed by intracellular immunofluorescence (IF) staining for CCHFV NP in moDCs at 48 hpi. As shown in Fig. 1C, CCHFV NP was observed in the cytoplasm of moDCs. Intracellular NP was not observed with IF in the other cell types nor could CCHFV NP be detected with Western blotting (data not shown).

To determine whether CCHFV induced a productive infection in target cells, the supernatants of all cell types were harvested at 48 hpi

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doi:10.1016/j.virol.2009.06.010

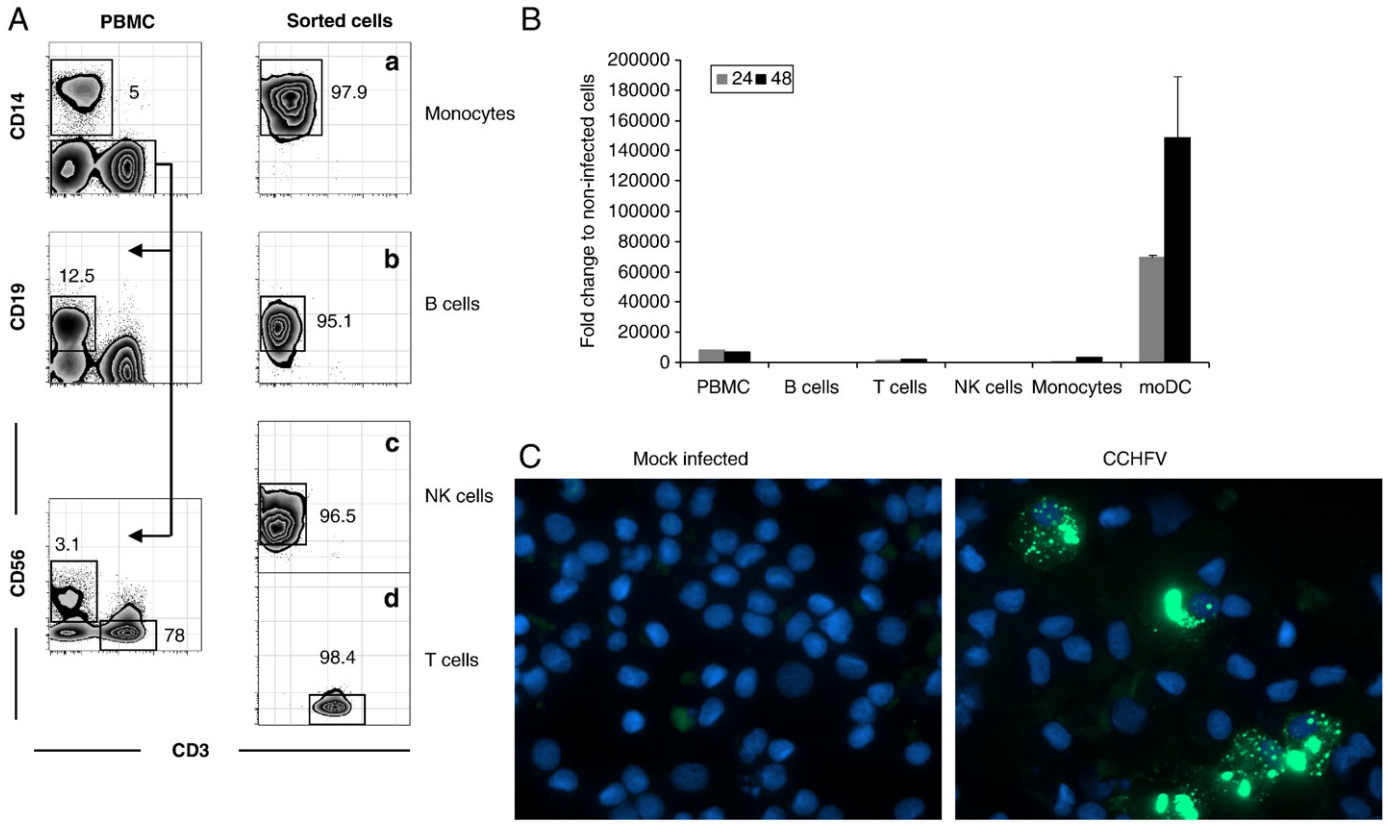

Fig. 1. moDCs are infected by CCHFV. (A) Total human PBMCs were stained for CD14, CD19, CD56, and CD3 and sorted using flow cytometry into four cell subsets consisting of B cells (CD19+), T cells (CD3+CD56−), NK cells (CD3−CD56+) and monocytes (CD14+). The purity of each of the sorted cell population was evaluated after sorting and was consistently >95%. (B) Total PBMCs, B cells, T cells, NK cells, monocytes and moDCs were infected with CCHFV at MOI 10. At 24 and 48 hpi cells were harvested for determination of CCHFV RNA levels. Results are shown as fold increase of mock infected cells within each time point. Only moDCs show an increase of RNA levels, whereas RNA levels in total PBMCs and all other PBMC subsets are much lower. (C) Mock infected and infected moDCs were fixed and stained for CCHFV NP 48 hpi. Infected cells show the characteristic cytoplasmic localization of NP.

and progeny viruses were quantified. Consistent with the data described above, and as depicted in Fig. 2, we found that the only cell types, which could be productively infected were moDCs.

Cytokine response of moDCs to CCHFV infection

Earlier studies have shown that elevated IL-6 and TNF-α levels are detected in sera of CCHFV infected persons and correlate positively to severity of disease (Ergonul et al., 2006; Papa et al., 2006). Given that CCHFV can infect moDCs, we were interested in determining whether

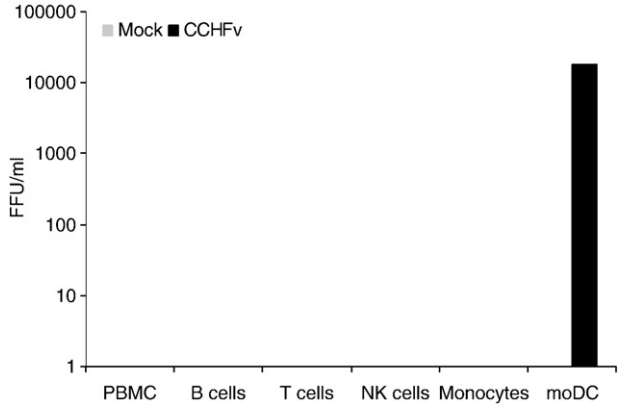

Fig. 2. moDCs are productively infected by CCHFV. Supernatants from 48 hpi infected or mock infected PMBCs and subsets were titrated for progeny virions. Progeny viruses were only detectable in moDCs.

the infection resulted in cytokine production. To address this question, we measured the secretion of TNF-α, IL-6, IL-10, IL-8, IL-19 and IL-1β following infection by CCHFV of moDCs derived from four naïve donors (termed A, B, C and D). Cell-free supernatants were collected at 48 hpi and assayed by ELISA for the previously mentioned cytokines. As shown in Fig. 3A consistently higher levels of TNF-α, IL-6 and IL-10 were measured in supernatants from infected moDCs compared to uninfected cells. However, there was no difference between infected and uninfected cells with regards to IL-8, IL-19 and IL-1β release (Fig. 3A and data not shown). LPS (1 μg/ml) was included as a positive control for all cytokines (data not shown). Relative Q-PCR analysis for CCHFV infection of moDCs from the same donors (Fig. 3B) verifies infection of moDCs.

Supernatants from infected moDCs activate endothelial cells

The role of the endothelium in viral hemorrhagic fevers (VHFs) has not been clarified, but it can be targeted in two ways. Either the activation and dysfunction is caused directly by virus infection and replication or the endothelium is targeted indirectly by virus-mediated host-derived soluble factors (Schnittler and Feldmann, 2003). Intercellular adhesion molecule 1 (ICAM-1) expression is increased upon endothelial activation, and it can therefore be used as a marker of activation (Hubbard and Rothlein, 2000). To address the role of secreted pro-inflammatory factors from infected moDCs in endothelial activation, we exposed confluent HUVECs to cell culture medium, LPS, or supernatants from uninfected and infected moDCs collected at 48 hpi. RNA was extracted from HUVECs 6 h after exposure and ICAM-1 mRNA relatively quantified with RT-PCR. As illustrated in Fig. 4A, endothelial cells respond to LPS treatment by increasing

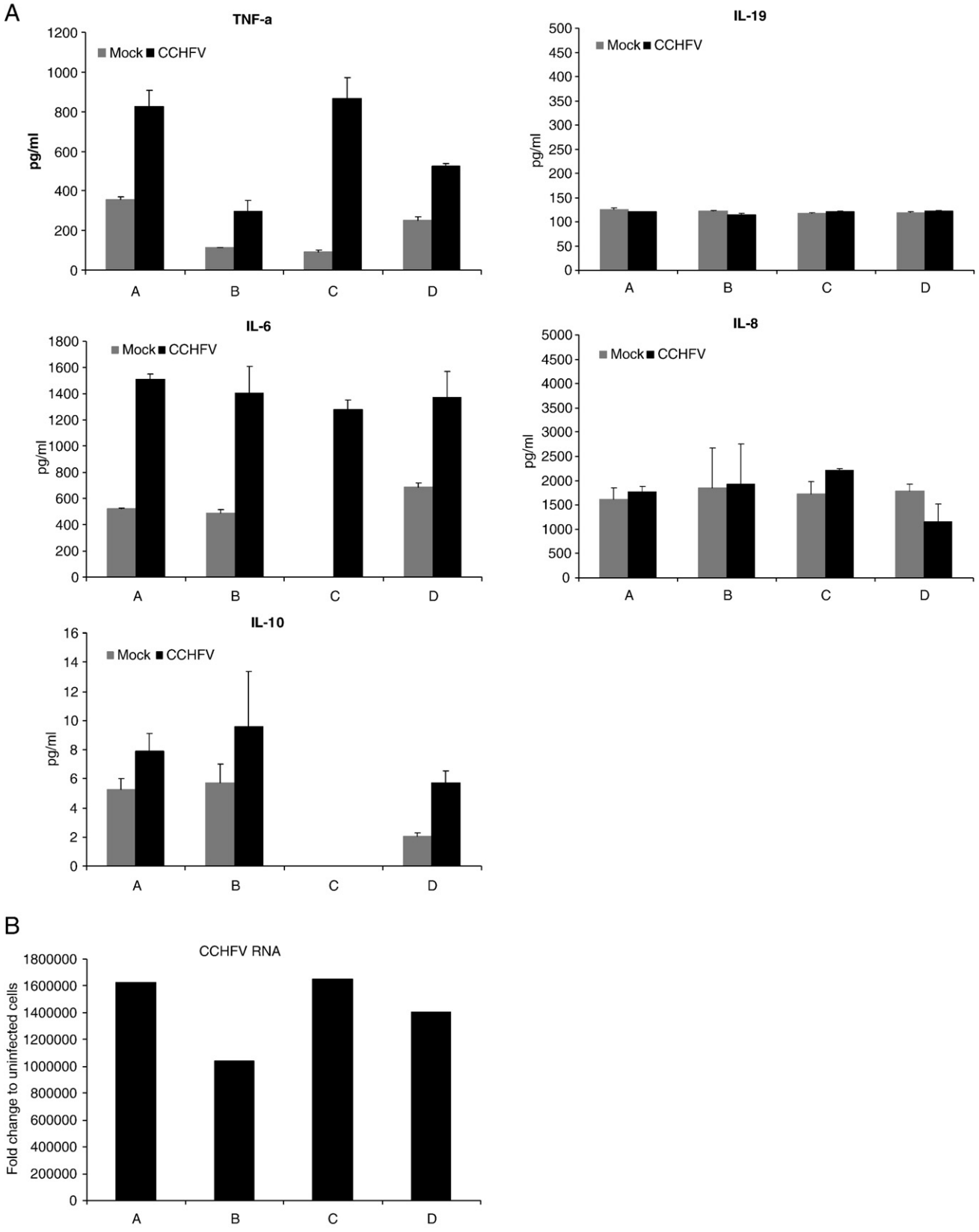

Fig. 3. Increased release of IL-6, IL-10 and TNF-α from infected moDCs. (A) Supernatants from mock infected and infected moDCs at 48 hpi were analyzed for TNF-α, IL-6, IL-10, IL-8 and IL-19 by ELISA. Infected moDCs seem to release higher levels of IL-6, IL-10 and TNF-α compared to uninfected cells, however no clear differences were observed with regards to IL-8 and IL-19 release. (B) CCHFV NP RNA levels for all four donors at 48 hpi are shown verifying infection.

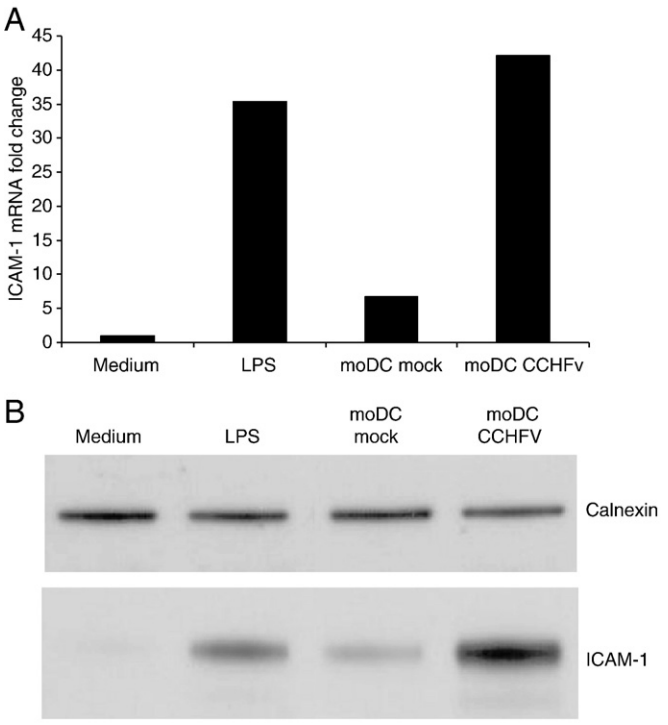

Fig. 4. Supernatants from infected moDCs activate endothelial cells. HUVEC cell layers were exposed for 6 h to cell culture medium (negative control), LPS 100 μg/ml (positive control) and to supernatants from mock infected and infected moDCs. Only moDC supernatants and LPS treatment activate endothelial cells as measured by ICAM-1 mRNA (A) and protein (B) up-regulation.

mRNA levels of ICAM-1 by more than 30-fold compared to the base line ICAM-1 mRNA levels in the negative control. Supernatants from CCHFV infected moDCs also activate endothelial cells as shown by a similar 30-fold increase of ICAM-1 mRNA. Exposure of endothelial cells to CCHFV alone does not cause ICAM-1 mRNA levels to increase after 6 hpi (data not shown). These results are verified by Western blotting (Fig. 4B).

Discussion

CCHF pathogenesis is poorly studied due to several reasons, (i) the virus is classified as a BSL-4 pathogen, (ii) currently no *in vivo* model exists and (iii) CCHF outbreaks are sporadic and very few of the endemic countries have the required safety facilities for working with infectious material.

The only published information regarding human target cells originated from post mortem studies of CCHF patients showing consistent infection of hepatic endothelial cells and macrophages (Burt et al., 1997). However, that study represents the end-point of a fatal infection and the initial cellular targets of CCHFV remain undefined. This is the first *in vitro* study to our knowledge, which focuses on characterizing the susceptibility of human blood cells to CCHFV infection and identifies immature moDCs as permissive to CCHFV infection and replication. This is in line with previous studies where several members of viral hemorrhagic fevers can infect moDCs (Bosio et al., 2003; Ho et al., 2001; Mahanty et al., 2003; Raftery et al., 2002).

In addition, we find an increased release of the cytokines IL-6, TNF-α and IL-10 from infected moDCs compared to uninfected cells. Two separate patient studies in Albania and Turkey indicate that IL-6 and TNF-α are the cytokines mostly induced during CCHF infection. In both studies, the levels of TNF-α correlated with disease severity (Ergonul et al., 2006; Papa et al., 2006). The role of IL-6 is more unclear since the Turkish study found a correlation with IL-6 levels and fatality (Ergonul et al., 2006), but the Albanian study found IL-6 to be elevated both in mild and severe forms of the disease casting some uncertainty as to the prognostic value of this cytokine (Papa et al., 2006). The role of IL-10 in CCHF disease severity is also unclear. In the Albanian study, there were high levels of IL-10 in the only patient that succumbed (Papa et al., 2002) however in the Turkish study IL-10 correlated negatively with disseminated intravascular coagulopathy (Ergonul et al., 2006). We also tested other molecules that could potentially play a role in pathogenesis of viral hemorrhagic fever viruses (IL-1β and IL-8) or attributed a role in tissue injury in sepsis (IL-19) (Baize et al., 2002; Hsing et al., 2008; Talavera et al., 2004). Here, in our *in vitro* study, we could not demonstrate any differences between CCHFV infected and uninfected moDC release of these molecules.

Other studies of hemorrhagic fever viruses show contradictory responses of moDCs and various patterns in cytokine responses to infection (Baize et al., 2002, 2009; Bosio et al., 2003; Bozza et al., 2008; Ho et al., 2001; Mahanty et al., 2003; Raftery et al., 2002; Scott and Aronson, 2008). The diverging responses to infection could possibly be one of the reasons for explaining the difference in molecular pathogenesis for VHFs. Our studies suggest that dendritic cells could contribute to the elevated cytokine response observed in CCHF patients; however there is need of an animal model to pinpoint the role of dendritic cells in CCHF pathogenesis.

Furthermore, we show that supernatants from infected moDCs activate endothelial cells by up-regulating ICAM-1 expression. The ICAM-1 up-regulation is not dependent on viral infection as infected endothelial cells do not respond after 6 hpi, indicating it is virally induced soluble mediators from moDCs that activates endothelial cells. One of the most obvious symptoms in VHFs is bleeding indicating endothelial dysfunction. This dysfunction could be caused by direct viral infection or indirect by activation of immunological and inflammatory pathways (Schnittler and Feldmann, 2003). It is now clear that Ebola viruses do not directly cause endothelial damage, and that the vascular leakage is likely a result of a deregulated immune response (Baize et al., 2002; Geisbert et al., 2003). What occurs in CCHF patients is not clear; however our previous study investigating the effect of *in vitro* infection on tight junction integrity supports the notion of indirect effect (Connolly-Andersen et al., 2007; Weber and Mirazimi, 2008). Likewise the expression of ICAM-I of endothelial cells upon exposure to moDC supernatants *in vitro* further supports an indirect effect, but more extensive studies are needed to clarify the interplay between the immune system and the endothelium to understand CCHF pathogenesis.

Materials and methods

Human peripheral blood cell isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy blood donors by collection of buffy coats provided by the blood bank at the Karolinska Hospital and following Ficoll-Paque (GE Healthcare) density gradient centrifugation. Following separation, cells were stained for CD14, CD56, CD3 and CD19 (all from BD Pharmingen). Monocytes, B cells, T cells and NK cells were isolated by sorting CD14+, CD19+, CD3+CD56−, CD56+CD3− cells, respectively, on a FACS Aria flow cytometer (BD Immunohistochemistry Systems). Following purification, samples were acquired on a FACS Aria and analyzed with FlowJo software.

In vitro differentiation of monocyte-derived dendritic cells

PBMCs were enriched for monocytes following plastic adhesion during 2 h at 37 °C. To obtain immature DCs, monocytes were further cultured for 6 days with RPMI 1640 medium containing 10% FCS, 2 mM

L-glutamin, 100 U/ml penicillin, 100 M streptomycin, 2% HEPES (all from Invitrogen Life Technologies) with 200 ng/ml GM-CSF (Peprotech) and 75 ng/ml r-IL4 (R and D systems).

Cell infection and stimulation

Cells were infected with CCHFV strain Ibar 10200 at an MOI of 10 or mock infected with cell culture medium and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 24 or 48 hpi. Following infection cells were centrifuged and supernatants were saved for further analysis. Cell pellets were re-suspended in medium and cells were treated to prepare for RNA isolation or IF staining to determine CCHFV infectivity.

Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, MD) were grown according to the manufacturer's instructions. Confluent HUVECs were exposed to supernatants from moDCs or treated with cell culture medium or 100 μg/ml LPS (Wollin et al., 1987). After 6 hour stimulation, cells were harvested and subjected to ICAM-1 determination by Q-PCR. All handling of the virus occurred in a BSL-4 laboratory at the Swedish Institute for Infectious Disease Control.

RNA isolation

Total RNA was isolated by chloroform extraction from Trizol treated cells. RNA extraction from the aqueous phase was performed using the Qiagen Mini Viral RNA kit according to the manufacturer's instructions and reverse transcribed with random primers yield cDNA.

Relative quantitative PCR

Quantification of ICAM-1, GAPDH and CCHFV NP cDNA was performed on the Applied Biosystems 7900 HT sequence detection system. Specific primers were designed for CCHFV NP: Forward primer 5′-GCCATGATGTATTTGCCTTTGA-3′, reverse primer 5′-CCAGTGAGCCATGAGCATGT-3′; ICAM-1: forward primer 5′-TGGCCCTCCATAGACATGTGT-3′, reverse primer 5′-TGGCATCCGTCAGGAAGTG-3′ and GAPDH: forward primer 5′-CAGCATCGCCCCACTTG-3′, reverse primer 5′-GAAATCCCATCACCATCTTCCA-3′. The expression of GAPDH served as an internal standard. The following formulas were applied for calculating results: $Ct = \text{gene } Ct - \text{GAPDH } Ct$ where Ct indicates the cycle threshold of GAPDH and gene of interest. Results were calibrated against a negative control and further analysed by the $2^{-\Delta ct}$ method.

Western blotting

Western blotting was performed as previously described (Connolly-Andersen et al., 2007). Briefly described, infected cells were lysed in lysis buffer and separation of polypeptides occurred in an SDS-PAGE gel. Electrophoresis was carried out at a constant current of 200 volts. Transfer of proteins occurred to a nitrocellulose membrane followed by blocking of the membrane in 5% milk in 0.1% Tween (PBS-T). Following blocking, the membranes were incubated with rabbit anti-Calnexin or rabbit anti-ICAM-1. After washing in PBS-T, the membranes were developed with Amersham ECL Plus Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol.

Immunofluorescence assay

Aliquots of moDCs were spun in the Shandon Cytospin 4 centrifuge (Thermo) on to slides. Slides were fixed with ice-cold 80% acetone. The cells were incubated with a primary rabbit polyclonal anti-CCHFV NP (Andersson et al., 2004) followed by anti-rabbit FITC-conjugated antibodies. DAPI (Sigma, St. Louis, MO) was added to stain cell nuclei. Slides were analysed by immunofluorescence microscopy. Pictures were obtained with a Hamamatsu digital camera (Wasabi 1.4 Hamamatsu; Photonics, GmbH, Germany).

Cytokine quantification

Supernatants from treated, infected or untreated cells were collected and assayed for concentrations of IL-1β, IL-6, IL-10, TNF-α (Mabtech, Sweden), IL-8 and IL-19 (R and D Systems, United Kingdom) by ELISA according to the manufacturer's protocol.

Virus titration assay

Progeny virions were determined as previously described (Connolly-Andersen et al., 2007). Briefly, Vero E6 cells were grown in 96 well plates and infected with serially diluted supernatants from infected or non-infected cells. The cells were fixed with 80% acetone and staining of CCHFV NP was performed as described above. Fluorescing foci were counted in a microscope, enabling calculation of progeny virus titers.

Acknowledgments

This work was supported by grants from the Swedish Medicine Council (To A.M, K2007-56X-20349-01-3). We thank William Adams for his technical help with isolating monocyte-derived dendritic cells, and Vithia Gunalan and Sarah Palmer for language revision.

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