Rapid Communication

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

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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 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. As shown in Fig. 1C, CCHFV NP was observed in the cytoplasm of infected 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.
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 the infection resulted in cytokine production. To address this question, we measured the secretion of TNF-α, IL-6, IL-10, 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.
In both studies, the levels of TNF-α (Ergonul et al., 2006; Papa et al., 2006). The role of IL-6 is more relevant in CCHFV infection and replication. This is in line with previous studies and TNF-α focuses on characterizing the susceptibility of human blood cells to CCHFV infection and replication. Two separate patient studies in Albania and Turkey indicate that IL-6 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.

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 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 consistent infection of hepatic endothelial cells and macrophages 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 consistent infection of hepatic endothelial cells and macrophages. 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 that 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 de-regulated 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-1 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% CO₂ 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’-GCCATGATGATTGCCCTTTGA-3’, reverse primer 5’-CCAGTGAGCACATGAGCATGTGT-3’; ICAM-1: forward primer 5’-TGGCCCTCATAGATCATGTGT-3’, reverse primer 5’-TGGCATCGTCAGGAAGTG-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 = gene Ct – GAPDH Ct where Ct indicates the cycle threshold of GAPDH and gene of interest. Results were calibrated using the ΔCt 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 (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.

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