Differential activation profiles of Crimean–Congo hemorrhagic fever virus- and Dugbe virus-infected antigen-presenting cells

Christophe N. Peyrefitte,1,2 Magali Perret,1 Stephan Garcia,1,2 Raquel Rodrigues,1 Audrey Bagnaud,1 Sandra Lacote,3 Jean-Marc Crance,2 Guy Vernet,1 Daniel Garin,2 Michèle Bouloy4 and Gláucia Paranhos-Baccalà1

1Emerging Pathogens Laboratory, Fondation Médecins, IFR128 BioSciences Lyon-Gerland, Lyon, France
2Virology Laboratory, Armed Forces Biomedical Research Institute (IRBA), Emile Pardé, Grenoble, France
3INSERM, P4 – Jean Médecins Laboratory, Lyon, France
4Institut Pasteur, Unité de Génétique Moléculaire des Bunyavirus, Paris, France

Crimean–Congo hemorrhagic fever virus (CCHFV) is a highly pathogenic, tick-borne member of the family Bunyaviridae and the genus Nairovirus. To better elucidate the pathogenesis of CCHFV, we analysed the host innate immune response induced in antigen-presenting cells (APCs) infected in vitro by CCHFV. Monocyte-derived dendritic cells (DCs) and macrophages (MPs) were both shown to be permissive for CCHFV and to replicate the virus, as monitored by genomic and antigenomic strand quantification. Virus replication was, however, controlled, corroborating an efficient alpha interferon-induced response. The upregulation of CD-83 and CD-86 indicated that CCHFV induced a partial maturation of DCs, which were also shown to activate the secretion of interleukin (IL)-6 and IL-8, but no tumour necrosis factor alpha (TNF-α). On the other hand, in MPs, CCHFV infection elicited a high IL-6 and TNF-α response and a moderate chemokine response. Nevertheless, when we compared these APC responses with those seen after infection with Dugbe virus (DUGV), a mildly pathogenic virus genetically close to CCHFV, we found that, in spite of some similarities, DUGV induced a higher cytokine/chemokine response in MPs. These results suggest that CCHFV is able to inhibit the activation of inflammatory mediators selectively in infection in vitro and that these differences could be relevant in pathogenesis.

INTRODUCTION

Crimean–Congo haemorrhagic fever (CCHF) was first documented comprehensively in 1944–1945 in Crimea, but the virus was isolated later and called Crimean hemorrhagic fever virus (Chumakov, 1945, 1974). As it was impossible to differentiate it from Congo virus, a virus isolated from a child in the Democratic Republic of the Congo, it has been designated Crimean–Congo hemorrhagic fever virus (CCHFV) (Casals, 1969). CCHFV belongs to the genus Nairovirus of the family Bunyaviridae (Elliott et al., 2000). This family of enveloped viruses with a tripartite, single-stranded RNA genome of negative polarity comprises more than 300 viruses grouped into five different genera: Orthobunyavirus, Hantavirus, Phlebovirus, Nairovirus and Tospovirus (Elliott et al., 2000). The different viruses of the genus Nairovirus are grouped into seven serogroups, all of which appear to be transmitted by ticks, including CCHFV, which is transmitted mainly by Hyalomma, Dermacentor, Rhipicephalus and Boophilus (Honig et al., 2004). Whilst the CCHFV life cycle involves wild and domestic animals (Zeller et al., 1994), humans become infected through tick bites, crushing ticks in the absence of gloves or contact with blood or tissues from viraemic patients or animals (Schwarz et al., 1995). Currently, CCHFV is considered to be the most geographically widespread tick-borne virus (Ergonul, 2007). Its epidemiology mirrors the geographical distribution of Hyalomma ticks in more than 30 countries throughout Africa, south-east Europe, the Middle East and Asia (Chinikar et al., 2008; Ergonul, 2006; Papa et al., 2008; Rai et al., 2008; Christova et al., 2009).
The human disease caused by CCHFV infection is usually very severe: after an incubation period of 3–7 days, the clinical spectrum starts with fever, headache, myalgia, nausea, vomiting and diarrhoea during the pre-haemorrhagic stage, and is followed by the haemorrhagic manifestations such as petechiae, haematemesis, melaena, haematuria and haemoptysis during the haemorrhagic phase, leading to a mortality rate of up to 50% (Ergonul, 2006; Swanepoel et al., 1987). During the course of the disease, biological parameters may help to predict the severity of the disease (Cevik et al., 2008). Fatal outcomes might be related to a high viral load (Cevik et al., 2007), disseminated intravascular coagulation (Swanepoel et al., 1989), somnolence, melaena and thrombocytopenia (Cevik et al., 2008).

Little is known about the role of the in vivo or in vitro immune response in the pathogenesis of CCHF (reviewed by Bray, 2007; Weber & Mirazimi, 2008). This has been hindered by the fact that no animal model of the disease is available, except for a newborn mouse model, useful mostly for virus isolation (Gowen & Holbrook, 2008; Tignor & Hanham, 1993). Recent studies indicated an increased number of peripheral blood natural killer cells (Yilmaz et al., 2008), as well as highly activated macrophages (Onguru et al., 2008), in patients with CCHFV infection. Immunohistochemical analysis and in situ hybridization studies on patient tissues showed that CCHFV infects mononuclear phagocytes, hepatocytes and endothelial cells (Burt et al., 1997). In vitro models have highlighted key points, such as alpha interferon (IFN-α) inhibiting CCHFV replication in pre-treated cell lines (Andersson et al., 2006) due to MxA antiviral activity (Andersson et al., 2004), or the delay in innate response activation due to CCHFV interference with the IRF-3 activation pathway in infected A549 cell lines (Andersson et al., 2008).

Human macrophages (MPs) and dendritic cells (DCs) act as a vital link between the early innate and later adaptive immune response and have been reported to probably play a prominent role in the pathogenesis of some haemorrhagic fever viruses, such as Lassa (Baize et al., 2006) and Ebola (Bray, 2007) viruses. In the case of CCHFV, it was recently reported that, among peripheral blood mononuclear cells, DCs are permissive to CCHFV and elicit a response by releasing cytokines, but there was no information on MPs (Connolly-Andersen et al., 2009). To better understand the innate response to CCHFV infection, we studied the ability of CCHFV to infect two types of human antigen-presenting cell (APC), MPs and DCs, in vitro. We analysed the phenotypic alterations induced by CCHFV infection, as well as the cytokine and chemokine secretion profile of these APCs. Then, we used this model to compare the response to CCHFV with that to Dugbe virus (DUGV), a mild human pathogen (Burt et al., 1996) among the closest genetically related nairoviruses to CCHFV (Honig et al., 2004). Our data indicated that the two viruses triggered similar immune responses in DCs and MPs, but a higher cytokine/chemokine response in DUGV-infected MPs. This suggests that these differences might be relevant for pathogenesis.

RESULTS

MPs and DCs are susceptible to infection with CCHFV

In general, bunyaviruses infect many types of cell. However, CCHFV growth is somewhat limited even in the most permissive cells: in our hands, Vero cells are among the most sensitive cells, but maximum titres of approx. 10^6 focus-forming units (f.f.u.) ml^{-1} were reached at day 3 post-infection (p.i.) when cells were infected at an m.o.i. of 0.01. During human infection with arboviruses, DCs and MPs are supposed to be the primary target cells. Therefore, the susceptibility of these cells to infection with CCHFV was tested by using immature DCs or MPs derived from monocytes isolated from three different healthy blood donors. Each experiment presented in this work represents results obtained independently from three batches of cells, each test being performed in duplicate. CCHFV infection was carried out at three m.o.i.s, i.e. 0.01, 0.1 and 1.0 f.f.u. per cell. Expression of virus antigens was monitored by immunofluorescence analysis (IFA) (Fig. 1a; Supplementary Fig. S1, available in JGV Online) and the virus produced in the supernatant was titrated (Fig. 1b). It should be noted that, after the 1 h adsorption period, residual or desorbed virus was eliminated by abundant washing of the cell monolayer. Despite this careful washing step, we cannot exclude the possibility that a few virus particles could still be desorbed later and detected at 6 h p.i.

The virus released in the medium was detected as soon as 6 h p.i. in both DCs and MPs infected at the highest m.o.i. The virus growth curve (Fig. 1b) indicated that virus production peaked at 24 h p.i. and, at that time, expression of virus antigens was clearly detected by IFA in a significant fraction of the cell population (Fig. 1a; Supplementary Fig. S1). When cells were infected at a lower m.o.i., the virus yield was lower, but the kinetics of virus production had the same profile. It appears that MPs were more permissive than DCs: the percentage of infected MPs was higher than DCs when infected at the same profile. It appears that MPs were more permissive than DCs: the percentage of infected MPs was higher than DCs when infected at the same m.o.i. (e.g. m.o.i. = 1.0: 7.4 × 10^4 f.f.u. ml^{-1} in MPs vs 3.6 × 10^4 f.f.u. ml^{-1} in DCs). At 48 h p.i., the virus yield and percentage of infected cells decreased, but the effect was more pronounced at 72 h p.i., a time at which infectious CCHFV could not be titrated in the maintenance medium. This result was surprising, considering that infectious virus had been produced earlier, is relatively thermostable and should remain infectious in the maintenance medium. We hypothesized that the disappearance
of infectious virus at late times might be due to the phagocytic activities of these cells. It may also be that the IFN secreted in the medium (see the following paragraph, Discussion and Fig. 4) inhibits plaque formation in the titration assay.

To demonstrate unambiguously that CCHFV replication did occur, we established a quantitative, strand-specific RT-PCR detecting either the genomic or the antigenomic strands of the S segment. Detection of the genomic and antigenomic strands was performed by using RNA extracted from the infected cells. The kinetic curve (Fig. 1c) showed that the virus genome was released rapidly into the extracellular medium and increased progressively or remained as a plateau at late times p.i. We assayed also the antigenomic S RNA synthesized in infected cells (Fig. 1d). As expected, in the control, consisting of cells infected with UV-inactivated CCHFV, this molecule, which is a replicative intermediate, was not detected, even at late times p.i. However, high amounts were detected at 24 h in DCs and as early as 6 h in MPs infected at a high m.o.i. The number of antigenome molecules declined slightly at 48 and 72 h p.i. in both MPs and DCs, suggesting that virus replication was inhibited. One can note that, surprisingly, the virus genomic strand is detected earlier than the antigenome. There is one possible explanation: the genomic molecules detected at 6 h p.i. correspond to the input, which probably contains a large amount of non-infectious particles. Altogether, these data indicate that CCHFV was able to replicate in both DCs and MPs.

CCHFV elicits a partial maturation of DCs

The maturation of DCs is essential for the initiation of the immune response. After infection or uptake of antigens, DCs initiate responses via the secretion of chemokines and pro-inflammatory cytokines and the upregulation of a variety of co-stimulatory and chemokine receptors. To examine whether CCHFV induces the maturation of DCs, expression of the surface proteins CD-40, CD-80, CD-83, CD-86 and HLA-DR was evaluated by using flow cytometry. The expression levels of CD-40, CD-83 and CD-86 increased significantly with a dose–response effect, according to the m.o.i. (Fig. 2a). The CD-40 upregulation induced by CCHFV at an m.o.i. of 1.0 was shown to be 1.56-fold higher than in the mock control. The results shown in Fig. 2(a) represent cells collected at 24 h p.i., as the response did not change significantly during the 3 days of the experiment (not shown). Of note, UV-inactivated CCHFV had no effect on CD-40 expression regulation, whereas CD-86 and CD-83 were upregulated to a level similar to that induced by replicating virus at an m.o.i. of 0.1. With regard to CD-80 and HLA-DR, their expression was relatively high in mock-infected cells, but was not modulated after CCHFV infection (not shown).

Profile of inflammatory mediators released by CCHFV-infected DCs and MPs

As both DCs and MPs were permissive to CCHFV infection, we investigated whether they were functional for the release of some crucial mediators in the pathogenic cascade, the pro-inflammatory cytokines interleukin (IL)-6, IL-8, IL-10 and tumour necrosis factor alpha (TNF-α). The maintenance medium of the mock- or CCHFV-infected DCs was collected at 24, 48 and 72 h p.i. and the different cytokines were titrated by using ELISA. A significant level of IL-6 and IL-8 was secreted by CCHFV-infected DCs at 24 h p.i. (Fig. 2b) and was stable over the 3 days of the experiment (data not shown). Of note, inactivated CCHFV induced a high IL-8 secretion, comparable to that induced by the replicating virus, but no IL-6 secretion. In contrast to IL-6 and IL-8, expression of TNF-α and IL-10 was not upregulated by CCHFV (data not shown).

Regarding MPs and based on the well-defined role of chemokines and cytokines in the initiation of the immune response...
response, we analysed the pro-inflammatory mediator profile of cytokine/chemokines released: TNF-α, IL-6, MCP-1, IP-10, RANTES and MIP-1α. As in the case of DCs, the supernatant of mock- or CCHFV-infected MPs was collected at various times p.i. and the mediators released were analysed by ELISA. The level of cytokines was stable over the 3 days of the kinetics experiment; therefore, only the results obtained at 24 h p.i. are reported in Fig. 3. TNF-α and MCP-1 were highly and stably produced. IP-10, IL-6, RANTES and MIP-1α were also secreted in CCHFV-infected MPs, but to a lesser extent, as the levels were about 100-fold lower than those in lipopolysaccharide (LPS) controls (P<0.05). Of note, a dose–response effect was observed for TNF-α, IP-10 and IL-6. UV-inactivated CCHFV induced the secretion of MCP-1 to a level even higher than the mock control. The other pro-inflammatory mediators were not modulated by the inactivated virus.

**CCHFV infection in DCs and MPs activates IFN-α expression**

The type I IFN response was investigated, due to its key role in the early antiviral cell response. In both DC and MP supernatants, the IFN-α secretion level was high (2066 pg ml⁻¹ for DCs and 5359 pg ml⁻¹ for MPs) and a dose–response effect was observed, according to the m.o.i. (Fig. 4a). The results shown in this figure are those obtained at 24 h p.i. However, IFN-α levels remained constant at later times and it is noteworthy that IFN-α was secreted relatively late during infection, as it was not yet detectable at 6 h p.i., confirming results reported by others (Andersson et al., 2008). In addition, UV-inactivated CCHFV induced a moderate but significant IFN-α production in DC (78.4 pg ml⁻¹) and MP (15 pg ml⁻¹) supernatants. No IFN-β was detected in the supernatant of infected DCs or MPs. This was confirmed by the absence of IFN-β mRNA in infected cells at 24 h p.i. (not shown).

To investigate further whether the IFN pathway was activated, the expression of some key effectors, such as MX-1 and OAS-1, was analysed by evaluating the amounts of the corresponding mRNAs by quantitative RT-PCR in both DCs and MPs infected by CCHFV (Fig. 4b, c). UV-inactivated CCHFV was shown to induce a low expression level of MX-1 and OAS-1 mRNAs in MPs, but high expression in DCs. Infectious virus activated the transcription of these antiviral genes, the levels of synthesis being constantly high and being higher in DCs than in MPs (P<0.05).

**Comparison of the responses of CCHFV- and DUGV-infected APCs**

To analyse further the potential impact of the observed modulations with regard to pathogenesis, we examined the effects of DUGV, a mildly pathogenic nairovirus related
closely to CCHFV (Burt et al., 1996; Honig et al., 2004), in
APCs and compared them with those induced by CCHFV.
DUGV was first isolated in 1964 from the Amblyomma
variegatum tick in Nigeria (Causey, 1970). Endemic in
most of the arid regions of Africa, it is one of the most
common tick-borne viruses found throughout this con-
tinent (Guilherme et al., 1996; Sang et al., 2006). This
nairovirus was selected for comparison with CCHFV
because, in contrast to the latter, DUGV causes only a
mild febrile illness, once described associated with
thrombocytopenia in humans (Burt et al., 1996).

DCs or MPs from the same batches used in the
experiments described above were infected with DUGV
in parallel experiments and their sensitivity and permissiv-
ity were assessed. Like CCHFV, DUGV replicated in these
cells but, when compared with CCHFV infection, we found
that DUGV grows to titres approximately 10 times higher
than CCHFV when infected at the same m.o.i. (e.g. at an
m.o.i. of 1.0, DCs produced 1.3 × 10^5 f.f.u. DUGV ml^{-1}, cf.
3.6 × 10^4 f.f.u. CCHFV ml^{-1}; MPs produced 7.4 × 10^5 f.f.u.
DUGV ml^{-1} vs 4.4 × 10^5 f.f.u. CCHFV ml^{-1}). In addition,
replication of DUGV in both cells decreased at late times
p.i., like CCHFV but more slowly: DUGV (but not
CCHFV) was still detected at 72 h p.i. (6 × 10^5 f.f.u. ml^{-1}
in DCs and 2 × 10^4 f.f.u. ml^{-1} in MPs).

We then analysed the immune response triggered by
DUGV in these APCs. Like CCHFV, DUGV activated

DISCUSSION
We analysed here the response of two different types of
APC, DCs and MPs, induced by the highly pathogenic
CCHFV infection. We also compared this cellular response
with that induced by DUGV, a mild or non-pathogenic

Fig. 3. Cytokine and chemokine production by CCHFV-infected MPs. The soluble mediators were quantified in pg ml^{-1} by
using ELISA. The bars represent means ± SD of three independent experiments.
virus for humans. This model could help to better identify key elements of CCHFV pathogenicity at the cellular level during the very early steps of the virus infection.

The two types of APC were shown to be susceptible and permissive to CCHFV, MPs being more permissive. The CCHFV load in the supernatant peaked at 24 h p.i., but became almost undetectable in both DCs and MPs at 72 h p.i. when assayed for infectious virus. This is in contrast with the assay detecting genomic RNA, which showed that the RNA molecules increased between 24 and 72 h (Fig. 1c). Therefore, it seems likely that IFN-α, which was secreted in relatively high amounts (Fig. 4a), inhibited the development of plaques in the f.f.u. assay. Moreover, quantification of the CCHFV antigenomic strand showed a slight decrease in synthesis, strongly suggesting negative control of CCHFV replication, most probably due to the antiviral effect of IFN-α produced by the infected cells. The production of CCHFV in both DCs and MPs suggests that, in vivo, these two types of cell may play a key role in the

Table 1. Differences observed in DC and MP activations during CCHFV or DUGV infection

<table>
<thead>
<tr>
<th></th>
<th>Mock</th>
<th>LPS (10 ng ml⁻¹)</th>
<th>CCHFV m.o.i. 1.0</th>
<th>DUGV m.o.i. 1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-83 MFI ratio</td>
<td>1</td>
<td>5.1 ± 0.6</td>
<td>2.67 ± 0.31</td>
<td>1.70 ± 0.19</td>
</tr>
<tr>
<td>CD-86 MFI ratio</td>
<td>1</td>
<td>10.71 ± 1.25</td>
<td>9.29 ± 1.05</td>
<td>2.45 ± 0.27</td>
</tr>
<tr>
<td>IL-6 (pg ml⁻¹)</td>
<td>0</td>
<td>210.1 ± 90.3</td>
<td>7.5 ± 13.2</td>
<td>264.8 ± 2.4</td>
</tr>
<tr>
<td><strong>MPs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg ml⁻¹)</td>
<td>0</td>
<td>660.8 ± 284.1</td>
<td>26.1 ± 5.9</td>
<td>234.0 ± 95.9</td>
</tr>
<tr>
<td>RANTES (pg ml⁻¹)</td>
<td>3.6 ± 2.2</td>
<td>1108 ± 465.4</td>
<td>13.3 ± 5.7</td>
<td>760.3 ± 281.3</td>
</tr>
<tr>
<td>IP-10 (pg ml⁻¹)</td>
<td>0</td>
<td>1521 ± 281.3</td>
<td>22.4 ± 5.2</td>
<td>739.0 ± 161.8</td>
</tr>
<tr>
<td>MIP-1α (pg ml⁻¹)</td>
<td>14.1 ± 6.2</td>
<td>1540 ± 585.2</td>
<td>59.6 ± 15.6</td>
<td>1974.2 ± 416.8</td>
</tr>
</tbody>
</table>
pathogenesis of this nairovirus at the very early stages of infection (Bray & Geisbert, 2005; Bray, 2007). Indeed, after a tick bite, CCHFV-infected APCs may host a first round of virus replication, facilitating a subsequent systemic dissemination of the virus (Gowen & Holbrook, 2008) and contributing to the high CCHF burden in the bloodstream, which may have an impact on the outcome of the disease (Cevik et al., 2007).

The maturation of DCs is crucial for the initiation of the immune response and is manifested by the expression of different surface membrane markers. We found that CCHFV was able to trigger DC upregulation of CD-86 and CD-83, but had a moderate effect on CD-40 and no effect on either CD-80 or HLA-DR, suggesting that it induced a sub-optimal maturation. When we compared it with other haemorrhagic fever viruses, it appears that each virus activates a specific pattern of surface markers on infected DCs: in the case of Lassa virus, DCs did not mature (Baize et al., 2004), and in the case of Ebola virus, CD-86 and HLA-DR expression increased, whereas that of CD-83 did not (Bosio et al., 2003). Interestingly, DCs infected by Ebola virus or Marburg virus failed to activate lymphocytes (Bosio et al., 2004). Although the profile of DC maturation is different between Ebola virus and CCHFV, both viruses induced a partial maturation of DCs; thus, it is tempting to assume that CCHFV-infected DCs are also unable to induce an efficient adaptive immune response. This might be correlated with the generalized suppression of adaptive immunity observed in patients with fatal disease.

To analyse essential elements of the innate immunity elicited in response to virus infection (Katze et al., 2002), we investigated whether IFN-α and IFN-β were expressed by the in vitro-infected APCs. In our model, IFN-β was not detected in supernatants of CCHFV-infected APCs. This result is in accordance with the strategy of CCHFV to evade the innate immune response through the cleavage of triphosphates at its RNA 5’ end (Habjan et al., 2008). In contrast, IFN-α was highly secreted during the 3 days of the study. Moreover, CCHFV-infected APCs were able to produce a sufficient amount of IFN-α to trigger the antiviral pathway and activate the transcription of OAS-1 and MX-1 mRNA, which was demonstrated previously to inhibit CCHFV replication (Andersson et al., 2004, 2006). This also adds support to the role of IFN on the transient production of infectious CCHFV in the maintenance medium of infected APCs (Fig. 1b). This situation is different from those encountered in Lassa virus infection, during which infected MPs induced a moderate IFN response whilst DCs did not (Baize et al., 2006), or in Ebola and Marburg virus infections, during which DCS and MPs did not produce any IFN-α (Bosio et al., 2003; Gupta et al., 2001). In the latter case, this is probably due to the IFN-antagonistic effect of the VP35 protein (Basler et al., 2000). So far, in CCHFV, the existence of viral proteins counteracting IFN activation, such as an NSs-like protein specific to bunyaviruses or the OTU domain of the L protein (Frias-Staheli et al., 2007), is still putative.

The pro-inflammatory cytokines expressed by DCs and MPs were also analysed, as they represent other elements of the response linked to pathogen elimination (Mohamadzadeh et al., 2006). Here, we observed that DCs infected in vitro by CCHFV produced a significant but moderate amount of IL-6, high amounts of IL-8 and no TNF-α. This is different from the data reported by Connolly-Andersen et al. (2009) showing that, when infected at an m.o.i. of 10, DCs produced high amounts of IL-6 and TNF-α, but not IL-8. Except for the m.o.i. and the source of the monocytes, these differences are unexplained. Recent clinical studies showed a higher level of IL-6 and TNF-α in CCHF patients with fatal outcome compared with non-fatal patients (Ergonul et al., 2006; Papa et al., 2006), suggesting that the haemorrhages associated with CCHF may be related to the high level of pro-inflammatory cytokine production (Bray, 2007; Connolly-Andersen et al., 2007). In our study, we found that CCHFV-infected MPs may contribute to pro-inflammatory cytokine production, as they secreted a high level of TNF-α and a moderate level of IL-6.

To understand better the impact of APCs on pathogenesis, we thought it was important to compare the data obtained with those for DUGV, a related but non-pathogenic virus. The two viruses both led to productive infections in the APCs. The DCs and MPs shared some modulation responses when infected with the two studied nairoviruses; however, two major differences were observed. Firstly, the expression levels of CD-83 and CD-86 were shown to be higher in CCHFV-infected DCs than in DUGV-infected DCs, suggesting a higher activation by CCHFV than by DUGV. Secondly, some of the mediators, IL-6, MIP-1α, IP-10 and RANTES, were secreted at a lower level in CCHFV-infected APCs than in DUGV-infected ones. The more striking difference was observed in MPs, where the level of expression was approximatively 30–50-fold higher after DUGV infection than after CCHFV infection. These findings suggest that, compared with DUGV, CCHFV exerts a selective inhibition especially on MP activation and, consequently, on their maturation during in vitro infection. Specific inhibition profiles have been described for other viruses: Lassa virus was shown to inhibit MIP-1α mRNA production in DCs (Baize et al., 2004), as well as IL-8 and TNF-α in monocytes/macrophages (Lukashevich et al., 1999). Langat virus was also able to selectively inhibit IL-12 but not TNF-α production in DCs (Robertson et al., 2009). Ebola virus inhibits the in vitro production of IL-6, IL-10 and RANTES in DCs, but not in adherent monocytes (Bosio et al., 2003). Moreover, in vivo, Ebola virus-infected macaques displayed high levels of various cytokines (IFN-α, MIP-1, TNF-α and MCP-1) in blood, but not IL-8 or IL-10 (Geisbert et al., 2003; reviewed by Bray, 2007).

Altogether, these results showing similarities in the cellular responses to the two nairoviruses, despite their different
clinical outcome, were unexpected. However, we observed differences that, temptingly, may offer elements to explain the pathogenesis of the diseases. Here, we focused on DCs and MPs, but this does not exclude the possibility that other cells, such as Kupffer or plasmacytoid cells, might be involved in the pathogenic cascade. Taken together, our data indicate the complex ability of these viruses to converse specifically with cells responsible for the innate immune response, suggesting a specific host signature in response to DUGV and CCHFV.

**METHODS**

**Virus.** All work with infectious viruses was carried out in a biosafety level 4 (BSL-4) facility for CCHFV and in a BSL-2 facility for DUGV. CCHFV (strain IbAr 10200) and DUGV (both obtained from the Pasteur Institute) were passaged in Vero (ATCC CRL-1586) cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 2% fetal calf serum (FCS) (BioWest). The supernatant was used as the virus stock (10^6 f.f.u. ml\(^{-1}\)). Absence of mycoplasma was confirmed by using a Mycoalert kit (Lonza). To produce replication-deficient virus, virus stock aliquots were inactivated by UV light [UV Mineral light lamp (model UVG-54), 254 nm; UVP, Inc.] at a distance of 1 cm on ice for 20 min. The absence of infectivity in the inactivated virus was then controlled by infecting 250,000 cells with 250 μl pure virus suspension in a 12-well plate and showing no f.f.u.

**Titration.** Vero cells were incubated in a 12-well plate (BD) with 250 μl of serial 10-fold dilutions of CCHFV- or DUGV-infected cell supernatants at 37 °C, 5% CO\(_2\) for 1 h in duplicate. Carboxymethylcellulose (CMC; 2.5%; Sigma-Aldrich) in DMEM/2.5% FCS was added and plates were incubated at 37 °C, 5% CO\(_2\) for 5 days. After CMC was removed with care, Vero cells were then fixed by using a 3.7% solution of formaldehyde (Sigma) in PBS (Invitrogen) at room temperature for 30 min, washed twice in PBS and permeabilized with 0.5% Triton X-100 (Sigma)/PBS at room temperature for 5 min. Vero cells were incubated by using an in-house hyperimmunized mouse ascitic fluid specific to CCHFV, kindly provided by Hervé Zeller/Philippe Marienneau (French Reference Centre for Haemorrhagic Fever Viruses, Pasteur Institute, Lyon, France) in 1% BSA (Sigma)/PBS solution at room temperature for 1 h, washed three times, then incubated in a peroxidase-conjugated goat anti-mouse antibody/1% BSA/PBS solution for 1 h at room temperature. Infected cell foci were revealed with diaminobenzimide (Sigma) and counted.

**Cells and infection.** Monocytes were isolated from a healthy blood donor (French National Institute of Blood Transfusion, Lyon, France) by using Ficoll-Paque PLUS and Percoll following the manufacturer’s protocol (GE Healthcare). Purified monocytes were cultured for 5 days either in an M-CSF (macrophage colony-stimulating factor; 20 ng ml\(^{-1}\); PeproTech)/10% FCS RPMI (Invitrogen) solution to generate MPs, or in an IL-4 (1000 IU ml\(^{-1}\); PeproTech)/GM-CSF (granulocyte-macrophage colony-stimulating factor; 2000 IU ml\(^{-1}\); PeproTech)/10% FCS RPMI solution to generate immature DCs.

MPs and DCs were harvested, seeded in a 12-well plate (10^6 cells per well) and infected with CCHFV or DUGV at an m.o.i. of 1.0, 0.1 or 0.01 (f.f.u. per cell) or incubated with LPS (10 ng ml\(^{-1}\); Sigma)/inactivated virus/supernatant from non-infected Vero cells (mock) at 37 °C, 5% CO\(_2\) for 1 h. This moment was considered as time 0, and the time course started from that point. The cells were washed gently three times with RPMI (Invitrogen), then incubated at 37 °C, 5% CO\(_2\), MPs, DCs and their corresponding supernatants were harvested at 6, 24, 48 and 72 h p.i. and centrifuged at 3000 g for 5 min. Supernatants were stored at −20 °C until use; cells were processed immediately.

**Indirect immunofluorescence (IFA).** MPs and DCs were fixed on a 12-well slide (Sigma), fixed with 4% paraformaldehyde/PBS, washed three times in PBS, permeabilized with 0.1% Triton X-100 PBS and then incubated in a 1% BSA/3% Ab serum (human serum, to saturate Fc\(\gamma\) receptors)/PBS solution for 10 min to saturate Fc\(\gamma\) receptors. The cells were incubated with a mouse hyperimmune ascitic fluid specific to CCHFV or DUGV in a 1% BSA/PBS solution at room temperature for 30 min, washed twice using PBS, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) in a 1% BSA/PBS solution at room temperature for 30 min. After three washes with 1% BSA/PBS solution, the cells were counterstained using Evans blue (Sigma), mounted, examined by using a UV–fluorescence microscope (Leica) and analysed by using MetaMorph v7.5 software (Wellcome Trust).

**Quantification of CCHFV antigenomic strands.** Briefly, RNAs from CCHFV-infected cells were extracted from cell pellets by using an RNeasy mini kit (Qiagen) and the S antigenomes were then quantified by using a quantitative RT-PCR developed in this work. A reverse-transcription step used 2 μl CCHF-rev primer [5’-TGGTGGCCTCCACACAGAAGCA-3’ (368–280), according to GenBank accession no. DQ211646)] at 10 μM in a mix containing 5 μl RNA extracts denaturated for 10 min at 65 °C, then mixed with 0.1 M dithiothreitol, 1 U RNase inhibitor μl\(^{-1}\) (Promega), 10 mM dNTPs, 3 mM MgCl\(_2\), 1 μl ImProm-II reverse transcriptase (Promega) and 4 μl of its buffer for a 1 h run at 42 °C. The enzyme was then inactivated for 15 min at 70 °C. CDNA (5 μl) was then mixed with 2 μl reaction mix provided with an LC Fast start DNA Master Hybridization Probes kit (Roche Diagnostics), 2 μl MgCl\(_2\), 25 mM, 1 μl each of primers CCHF-for [5’-TTCCAAAATGGCCAGTGCC-3’ (197–214)] and CCHF-rev at 10 μM, 0.4 μl CCHF probe [5’-(FAM)-ATGATGCACAAAGGATTCATCTCAAGCG-(TAMRA)-(phosphate)-3’ (positions 219–247 in the S antigenome)] at 25 μM. After an initial step for 10 min at 95 °C to activate the Taq polymerase, the cdNA was amplified during 40 PCR cycles of 15 s at 95 °C and 1 min at 60 °C. A standard curve was obtained by using an in vitro transcript from the pGEM-T Easy vector (Promega) containing the same CCHFV PCR fragment.

Genomic strands were quantified by using the same primers and probe in a one-step RT-PCR assay.

**Flow cytometry.** To evaluate the expression of cell-surface molecules, MPs and DCs were resuspended gently in a 1% Ab serum and 0.01% azide (Sigma)/PBS solution for 5 min, then incubated at room temperature for 5 min with different monoclonal antibodies conjugated to FITC (CD-80, HLA-DR; Becton Dickinson) or phycoerythrin (PE; CD-40, CD-83, CD-86; Immunotech) at the concentrations recommended by the manufacturer. Isotypic controls with irrelevant FITC- or PE-conjugated antibodies (IgG2a; BD Pharmingen) were used as negative controls. The purity of the MPs and CDs was assessed by using PE-conjugated CD-1a, CD-3, CD-14, CD-20 and CD-56 antibodies (Immunotech): DCs express CD-1a but not CD-14, whereas MPs express CD-14. Cells were washed twice in 0.1% BSA, 0.05% azide PBS solution, fixed in a 3% paraformaldehyde/PBS solution, then analysed in a flow cytometer (FACSCalibur; BD Pharmingen) using the Expo 32 software (Applied Cytometry Systems). The results were expressed as mean fluorescence (MFI) ratios (infected-cell MFI/mock MFI).

**Cytokine and chemokine detection.** Supernatants of MPs and DCs were analysed to determine the amount of cytokines and chemokines.
Detection of mRNAs encoding proteins of the IFN pathway. The mRNAs encoding MX-1 or OAS-1 were extracted from cell pellets by using a Qiaqen kit and quantified by using quantitative RT-PCR commercial kits following the manufacturer’s protocols (Search-LC; Roche). The levels of mRNA were normalized according to the β-actin mRNA level, which was amplified in tandem with each of the tested mRNAs using a LightCycler (Roche). For each mRNA, the ratio value was obtained as follows: ratio of mRNA of interest = 2^(-[(C_t gene of interest - C_t actin)^mock] - (C_t gene of interest - C_t actin)^mock).

Statistical analysis. Student’s t-test was used to compare two sets of data, with a P-value of <0.05 considered significant. SD was determined by using Microsoft Excel.

ACKNOWLEDGEMENTS

We thank Danielle Gratier and Corinne Rothlisberger for their excellent technical assistance, Hervé Zeller and Philippe Marianneau for providing mouse ascitic CCHFV antibodies and Sylvain Baize for fruitful discussions. We thank Mary O’Brien and Bernard Souberbielle for critically reviewing the manuscript. This work was supported by research grants from the Service de Santé des Armées, the Délégation Générale pour l’Armement, the ARAMI association, bioMérieux SA and the Fondation Mérieux. Part of this work was presented during the International Meeting on Emerging Diseases and Surveillance in Vienna, Austria, 13–16 February 2009.

REFERENCES


33. macaques: evidence that dendritic cells are early and sustained targets of viral proteases evade ubiquitin- and ISG-15-dependent innate immune responses. Cell Host Microbe

198. Journal of General Virology

Lassa and Mopeia virus replication in human monocytes/

Katze, M. G., He, Y. & Gale, M., Jr (2002).


