Crimean–Congo hemorrhagic fever virus infection is lethal for adult type I interferon receptor-knockout mice

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Crimean–Congo hemorrhagic fever virus (CCHFV) poses a great threat to public health due to its high mortality, transmission and geographical distribution. To date, there is no vaccine or specific treatment available and the knowledge regarding its pathogenesis is highly limited. Using a small-animal model system, this study showed that adult mice missing the type I interferon (IFN) receptor (IFNAR\(^{-/-}\)) were susceptible to CCHFV and developed an acute disease with fatal outcome. In contrast, infection of wild-type mice (129 Sv/Ew) was asymptomatic. Viral RNA was found in all analysed organs of the infected mice, but the amount of CCHFV RNA was significantly higher in the IFNAR\(^{-/-}\) mice than in the wild-type mice. Furthermore, the liver of IFNAR\(^{-/-}\) mice was enlarged significantly, showing that IFN is important for limiting virus spread and protecting against liver damage in mice.

Crimean–Congo hemorrhagic fever virus (CCHFV) belongs to the family Bunyaviridae, genus Nairovirus, and is endemic in large parts of Africa, Asia, the Middle East and south-east Europe (Hoogstraal, 1979; Ergonul, 2006). CCHFV causes a severe human disease characterized by fever, prostration, severe haemorrhage and death, with mortality rates from 5 to 70% (Baskerville et al., 1981; Schwarz et al., 1997; Bakir et al., 2005). Seroepidemiological studies performed in endemic areas have shown that CCHFV is widespread among domestic livestock, as well as in wild-animal populations (Swanepoel et al., 1987; Burt et al., 1993; Mariner et al., 1995). The virus is usually transmitted to humans by bites from infected ticks of the genus Hyalomma or by direct contact with the blood or tissues of infected animals (Khan et al., 1997; Nabeta et al., 2004). Existing knowledge of the pathogenesis and basic virus biology of this virus is very limited for several reasons: (i) handling of the virus requires high-containment laboratories (BSL-4); (ii) outbreaks occur in endemic countries that have no facilities for performing basic and/or applied research; (iii) there is a lack of clinical specimens from patients, animals and ticks; and (iv) there is currently a lack of suitable animal models (Shepherd et al., 1989; Gonzalez et al., 1998; Swanepoel et al., 1998).

The existing knowledge concerning CCHFV pathology originates from autopsies and clinical findings. Increased capillary leakage is the hallmark of all haemorrhagic fevers, whereas the primary pathophysiological events during CCHFV infection appear to be leakage of erythrocytes and plasma through the vasculature into tissues (Weber & Mirazimi, 2008). Furthermore, endothelial damage can contribute to coagulopathy by deregulated stimulation of platelet aggregation, which in turn activates the intrinsic coagulation cascade, ultimately leading to clotting-factor deficiency, causing haemorrhages. However, the molecular mechanism behind the pathogenesis of CCHFV infection is largely unknown. There is currently a demand for a small-animal model system, not only for studying the pathogenesis of CCHFV, but also for the development of antivirals and vaccines.

Type I interferons (IFN-\(\alpha/\beta\)) have strong antiviral effects and CCHFV is one of the newest additions to the list of IFN-sensitive viruses. We have demonstrated previously that the pre-treatment of host cells with IFN-\(\alpha\) inhibits the replication cycle of CCHFV, most probably due to induction of MxA and other IFN-stimulated genes (Andersson et al., 2004). Studies in mice lacking type I or type II IFN receptors have shown clearly that type I IFN and type II IFN (IFN-\(\gamma\)) are generally essential for antiviral defence (Huang et al., 1993; Muller et al., 1994; Hwang et al., 1995). For example, infection of IFNAR\(^{-/-}\) mice with attenuated Rift Valley fever virus or Dugbe virus is lethal (Bouloy et al., 2001; Boyd et al., 2006). Based on this information, we were interested in studying whether IFNAR\(^{-/-}\) mice are sensitive to CCHFV infection.
In order to examine whether CCHFV causes disease in mice lacking type I IFN responses, we experimentally inoculated IFNAR$^{-/-}$ mice and included adult immunocompetent mice as controls. The experiments were carried out in adult (7- to 10-week-old) female 129 Sv/Ew and IFNAR$^{-/-}$ mice on an inbred 129 Sv/Ew genetic background (MTC/Karolinska Institutet, Sweden). During the experiment, the animals were housed in the animal room within the BSL-4 laboratory under climate-controlled conditions, with artificial 12 h light/dark cycles. The animals were kept in an Isocage system (Tecniplast) containing Mouse House (Tecniplast) and corn cob bedding (Cobex Hungaria Kft.), and fed standard rodent feed (SDS) and water ad libitum. Infection and manipulation of infected animals were performed in a class 2 biological safety cabinet.

Mice were injected intraperitoneally with 100 µl CCHFV (strain IbAr 2000; originally provided by Heinz Feldmann, Public Health Agency of Canada, Winnipeg, MB, Canada) diluted with Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) to $10^6$, $10^5$, $10^3$ or $10^1$ f.f.u. per mouse. Control mice were inoculated with DMEM (Invitrogen). In the first study, a group of five IFNAR$^{-/-}$ mice and a group of five 129 Sv/Ew mice were inoculated with $10^6$ f.f.u. CCHFV per mouse. During the second study, groups of three mice were inoculated with $10^5$, $10^3$ or $10^1$ f.f.u. CCHFV per mouse.

Health status was monitored twice daily. The first signs of clinical disease (laboured breathing and porphyry around the nostrils and eyes) were observed about 42 h post-infection (p.i.) in the IFNAR$^{-/-}$ mice infected with $10^6$ and $10^5$ f.f.u. per mouse. Symptoms were observed at 62–70 h p.i. in the IFNAR$^{-/-}$ mice infected with $10^3$ f.f.u. per mouse or lower doses of CCHFV. Four to six hours following the first symptoms, the health status deteriorated significantly and, unless euthanized, the mortality of the IFNAR$^{-/-}$ mice was 100%, independent of the amount of virus used for infection (Fig. 1). In contrast, no clinical symptoms were observed in CCHFV-infected 129 Sv/Ew mice during the experiment.

Mice showing signs of severe disease were anaesthetized and euthanized by exsanguination, together with groups of mice (both 129 Sv/Ew and IFNAR$^{-/-}$) inoculated with other doses of the virus as controls. Infected 129 Sv/Ew mice were also euthanized at 11 days p.i., which was the end point of the experiments. Following euthanasia, a visual inspection was carried out of internal organs and the liver was weighed. To analyse the replication of CCHFV, tissue samples from the liver, spleen, kidney, heart, brain and whole blood were homogenized in TRIZol (Invitrogen) for RNA extraction.

Extracted RNA was stored at $-80^\circ$C prior to RT-PCR. Real-time RT-PCRs were performed as described previously (Wölfel et al., 2007). Positive reactions, defined as cycle crossing points <35.00, were plotted against a standard curve (Wölfel et al., 2007) and the genome contents were calculated. The results presented in Fig. 2 are based on viral RNA, which is not equivalent to infectious particles. However, by comparing the concentration ml$^{-1}$ of the two fractions, we found the concentration of viral RNA to be approximately 10 times the concentration of infectious particles (data not shown).

As expected, IFNAR$^{-/-}$ and 129 Sv/Ew mice were permissive to CCHFV infection (Fig. 2a–f). The liver and the spleen were the organs that showed the highest levels of CCHFV RNA (Fig. 2a–f). At 48 h p.i., IFNAR$^{-/-}$ mice challenged with the highest dose of virus ($10^6$ f.f.u. per mouse) contained up to $5.3 \times 10^{11}$ (liver) and $6.2 \times 10^{10}$ (spleen) copies (g viral RNA)$^{-1}$. We observed 3–4 logs less viral RNA in liver and spleen in the IFN-competent group (129 Sv/Ew mice) infected with $10^6$ f.f.u. per mouse. IFNAR$^{-/-}$ mice challenged with $10^3$, $10^5$ and $10^1$ f.f.u. showed stepwise lower viral load in the analysed organs (Fig. 2a–f). In 129 Sv/Ew mice infected with $10^5$–$10^1$ f.f.u., the viral load in the blood was below the detection level. In IFNAR$^{-/-}$ mice with a survival period longer than 48 h (mice challenged with $10^3$ and $10^1$ f.f.u.), a time-dependent increase in viral load was observed in all analysed organs. IFNAR$^{-/-}$ mice euthanized following the onset of severe disease (after 48, 68 and 74 h) showed similar titres of viral RNA in the organs, independent of the dose of virus used for the challenge (Fig. 2a–f).

The dynamics of virus replication in 129 Sv/Ew mice did not follow the same pattern as observed in IFNAR$^{-/-}$ mice. At 48 h p.i., the viral load in 129 Sv/Ew mice was between 10- and 1000-fold lower than that in the IFNAR$^{-/-}$ mice challenged with the same dose of virus. Independent of the dose of virus used for the challenge, in 129 Sv/Ew mice at 68 h p.i., the viral load was above the detection level only in the spleen and liver (Fig. 2b, c), and at 11 days p.i.,
the viral load in all samples was below the detection level. As noted above, CCHFV-infected IFNAR\(^{-/-}\) adult mice developed severe disease and died within 48–74 h, depending on the dose of virus used for infection. The viral RNA load found in the different organs at different time points p.i. was correlated with the dose of virus used for the infection (Fig. 2a–f). This pattern was also observed in certain organs (liver and kidney) in the 129 Sv/Ew mice (Fig. 2c, d), but not in other organs. The highest viral load in both IFNAR\(^{-/-}\) and 129 Sv/Ew mice was observed in the
liver and the spleen, followed by kidney, brain, heart and blood. However, 129 Sv/Ew mice showed 10- to 10000-fold lower viral loads than IFNAR<sup>−/−</sup> mice. Virus titres increased continuously in IFNAR<sup>−/−</sup> mice until death and, interestingly, we observed that a count as low as 10 f.f.u. per mouse was lethal for IFNAR<sup>−/−</sup> mice.

We were able to establish infection in 129 Sv/Ew mice challenged with 10<sup>6</sup>, 10<sup>5</sup> and 10<sup>3</sup> f.f.u. but, in contrast to IFNAR<sup>−/−</sup> mice, the viral load decreased continuously and the virus was cleared at 68 h p.i. from the kidney, brain, heart and blood, and at 11 days p.i. from the liver and spleen (Fig. 2a–f). This meant that, in contrast to liver and spleen, the viral load in the blood of infected 129 Sv/Ew mice was negative at 68 h p.i. Possible explanations are that the level of viral RNA in the blood of these mice was below the detection level, or that there was no virus in these mice at that time. Taken together, these data suggest that IFNs play an essential role in protecting mice from disease following CCHFV infection.

It is known that CCHFV causes multiple organ failure and liver lesions, which vary from disseminated necrotic foci to massive necrosis in human (Swanepoel et al., 1989). Our data indicate that the liver is one of the main target organs for CCHFV replication in mice (Fig. 2c). Interestingly, post-mortem examination revealed a swollen and congested liver (Table 1) with well-defined haemorrhagic areas in IFNAR<sup>−/−</sup> mice inoculated with 10<sup>4</sup>–10<sup>5</sup> f.f.u. per mouse (see Supplementary Fig. S1, available in JGV Online). These mice, which survived longer than 48 h p.i., had statistically significantly (<i>P</i>&lt;0.05; Fisher’s test) enlarged livers compared with non-infected IFNAR<sup>−/−</sup> mice or with infected and non-infected 129 Sv/Ew mice (Table 1). This observation was most evident in IFNAR<sup>−/−</sup> mice challenged with 10<sup>5</sup> f.f.u. virus, where the liver represented 3.6, 4.6 and 5.4 % of the body mass at 48, 68 and 74 h p.i., respectively. However, we did not observe any increase in size or macroscopic changes in the liver of IFNAR<sup>−/−</sup> mice infected with high levels of f.f.u. In IFNAR<sup>−/−</sup> mice challenged with 10<sup>5</sup> f.f.u. virus, the size and macroscopic appearance of the liver remained unaffected at death. The results indicate that swelling of the liver is a response to virus replication, so the mice inoculated with the highest dose of virus might have died before liver damage had occurred.

It has previously been shown that CCHFV can cause lethal infection of newborn mice (Smirnova et al., 1973; Gonzalez et al., 1995). The infant-mouse model has been used to verify that ribavirin has beneficial effects for CCHFV-infected mice (Tignor & Hanham, 1993). Moreover, adult mice have been used in a vaccine experiment studying recombinant DNA vaccines against CCHFV (Spik et al., 2006). However, both models have clear disadvantages. The immune system in infant mice has not matured fully and, moreover, infant mice cannot be used in a vaccine study, while CCHFV infection of adult mice is asymptomatic and it is therefore not possible to study the protective effects of any vaccine in this animal model.

The present study demonstrates that CCHFV infection of adult IFNAR<sup>−/−</sup> mice, which lack the type I IFN receptor, causes rapid-onset symptoms and death in animals infected with as low a dose as 10 f.f.u. virus. The virus was found in all organs analysed and, importantly, we observed that the liver was clearly affected. The results suggest that the CCHFV/IFNAR<sup>−/−</sup> mouse model can be of use in future studies aimed at the development and evaluation of antiviral treatments and vaccines.

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### References

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### Table 1. Liver mass measured at different time points following infection with CCHFV

Presented data show the mean (three mice per group) and SD of liver mass expressed as a percentage of body mass; ND, not done. Statistical significance of the differences between groups was established by Fisher’s test.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Virus dose (f.f.u.)</th>
<th>Liver mass (percentage of body mass) ± SD</th>
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<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>68 h</td>
</tr>
<tr>
<td>IFNAR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>100 000</td>
<td>3.64 (± 0.06)</td>
</tr>
<tr>
<td>IFNAR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1000</td>
<td>5.02 (± 1.00)</td>
</tr>
<tr>
<td>IFNAR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>10</td>
<td>3.55 (± 0.12)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>3.59 (± 0.26)</td>
<td>ND</td>
</tr>
<tr>
<td>129 Sv/Ew</td>
<td>100 000</td>
<td>3.91 (± 0.09)</td>
</tr>
<tr>
<td>129 Sv/Ew</td>
<td>1000</td>
<td>3.55 (± 0.26)</td>
</tr>
<tr>
<td>129 Sv/Ew</td>
<td>10</td>
<td>3.74 (± 0.12)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>3.40 (± 0.28)</td>
<td>ND</td>
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