Infection of hepatocytes with 17-D vaccine-strain yellow fever virus induces a strong pro-inflammatory host response

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Yellow fever virus (YFV) causes serious disease in endemic areas of South America and Africa, even though a very well tolerated vaccine is available. YFV primarily targets the liver where as many as 80% of hepatocytes may be involved during infection. The objective of this project was to compare and contrast the cytokine response from hepatocytes infected with either wild-type (Asibi) or vaccine (17-D-204) strains of YFV, with the goal of identifying responses that might be correlated with disease severity or vaccine efficacy. We report here that PH5CH8 hepatocytes support a productive infection with both wild-type and vaccine-strain YFV. Infection with either virus resulted in elevated expression of several pro- and anti-inflammatory cytokines [interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10 and tumour necrosis factor-α] with a corresponding increase in transcription. Hepatocytes infected with vaccine virus had a more profound response than did cells infected with wild-type virus. Pre-stimulation of hepatocytes with IL-6 resulted in reduced viral titres, elevated concentrations of cytokines released from Asibi virus-infected cells and improved cell viability in cells infected with 17-D virus. Data reported here suggest that 17-D virus stimulates an appropriate antiviral inflammatory response in hepatocytes, while Asibi virus can attenuate the host response. These data identify potential mechanisms that are associated with increased virulence in wild-type virus infections and also provide clues towards potential immune-response limitations that may be associated with vaccine-related adverse events.

INTRODUCTION

Yellow fever (YF) is a disease that affects approximately 200 000 people worldwide and results in an estimated 30 000 deaths each year (World Health Organization, 2006). The causative agent of YF, yellow fever virus (YFV) (family Flaviviridae, genus Flavivirus), is an arthropod-borne virus endemic to tropical South America and sub-Saharan Africa. YFV poses a risk to those living in endemic areas, estimated at 900 million people, and travellers (Gardner & Ryman, 2010). YFV is a significant medical threat as reaching and maintaining sufficient vaccination coverage is challenging. Additionally, there are no medical therapies for the treatment of yellow fever.

YFV 17-D is a live attenuated virus approved for use as a vaccine and is described as being one of the most safe and efficacious vaccines in use today as >500 million doses have been administered since its development in 1937 (Monath et al., 2010; Theiler & Smith, 1937). Greater than 95% seroconversion has been reported in first-time vaccinees. A small fraction of vaccinees have developed severe adverse events following 17-D vaccination, with an apparent increase in incidence over the past several years (Bae et al., 2008; Belsher et al., 2007; Doblas et al., 2006; Gerasimon & Lowry, 2005; Monath, 2010; Whittembury et al., 2009). These cases appear to be associated with advanced age (>65 years) or a history of thymus disease (Bae et al., 2008). Severe adverse events are characterized as either yellow fever vaccine-associated neurotropic disease (YF-AND) or yellow fever vaccine-associated viscerotropic disease (YF-AVD). YF-AND patients typically resolve infection after a brief, severe disease that may include, but is not limited to, encephalitis. YF-AVD patients, however, typically progress to a disease state that resembles wild-type YFV infection resulting in the deaths of some of the patients. Until we have a better understanding of host factors associated with development of serious adverse events, it is important to identify host responses to the vaccine and wild-type viruses that might correlate with disease severity.

While several groups have published studies or case reports on cytokine responses following vaccination with 17-D (both normal-responders and severe adverse events) or...
infection with wild-type YFV, most of these reports are limited regarding the information given or cytokines examined (typically only one blood sample is available for testing after disease onset). Studies that have examined cytokine expression during wild-type infection or vaccination have suggested many different cytokines as having roles in YFV infection and disease progression including: interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, transforming growth factor (TGF)–β, tumour necrosis factor (TNF)-α and interferon (IFN)–γ. Bae et al., 2008; Belsher et al., 2007; Doblas et al., 2006; Gaucher et al., 2008; Gerasimon & Lowry, 2005; Hacker et al., 1998, 2001; Khaiboullina et al., 2005; Quaresma et al., 2006, 2007; Querec et al., 2006; Reinhardt et al., 1998; Santos et al., 2008; Silva et al., 2010; ter Meulen et al., 2004; van der Beek et al., 2002; Verschuur et al., 2004; Wheelock & Sibley, 1965).

The liver is the primary target organ during wild-type YFV infection, affecting as many as 80% of hepatocytes (Klotz & Belt, 1930). Post-mortem examination of YF liver shows four distinct pathologies: Councilman bodies (eosinophilic degeneration of hepatocytes and Kupffer cells), hepatocellular necrosis, focal necrosis and inflammation. The liver returns to normal histology without scarring in surviving cases (Klotz & Belt, 1930; Monath & Barrett, 2003). The mechanisms behind the pathological changes of the liver during wild-type YFV infection are unclear, although much of the pathology seen is potentially because of the host inflammatory response.

Aside from post-mortem examination, the YF liver is greatly underinvestigated. Considering that the liver is a primary target during YFV infection and its dysfunction during infection results in jaundice, probable dysregulation of the acute phase response (and thus coagulation abnormalities) and organ failure, it is important to understand how hepatocytes respond to YFV infection. The aim of this study was to compare and contrast viral growth and cytokine induction following hepatocyte infection with either wild-type (Asibi) or vaccine (17-D) YFV strains. We report here that PH5CH8 hepatocytes support replication of both viruses, and that several cytokines are upregulated and released during infection. Hepatocytes infected with 17-D virus responded by producing high concentrations of cytokines with extensive cell death, while Asibi virus-infected hepatocytes had a limited cytokine response unless the cells were pre-stimulated by IL-6. These data suggest that Asibi virus may limit intracellular control mechanisms, leading to higher viral titres and reduced host responses in PH5CH8 hepatocytes.

RESULTS

Asibi (wild-type) virus grows to higher titres in PH5CH8 hepatocytes than 17-D (vaccine) virus

Hepatocytes are a principal target of YFV infection; histological evidence demonstrates that they carry the bulk of viral antigen load upon post-mortem examination of livers from humans, hamsters and non-human primates following wild-type YFV infection, and in serious adverse-event vaccination patients (Chan et al., 2001; Monath et al., 1989; Sbrana et al., 2006). 17-D vaccinees usually develop a low level ($10^2$ p.f.u. ml$^{-1}$) viraemia 3–6 days post-vaccination that is short lived, but could possibly lead to hepatocyte infection (Reinhardt et al., 1998; Wheelock & Sibley, 1965). 17-D has also been shown to replicate in hepatoma cell lines (Huh-7) and primary hepatocytes (Anwar et al., 2009; Chan et al., 2001; Zhou et al., 2011). The study described here evaluated the growth kinetics of both Asibi and 17-D viruses in the non-neoplastic PH5CH8 human-hepatocyte cell line (Preiss et al., 2008). Both viruses were found to replicate efficiently; however, Asibi virus replicated to significantly ($P<0.05$) higher titres than 17-D virus in these hepatocytes (Fig. 1a). Infectious virus was detected from both Asibi and 17-D virus-infected hepatocytes until 120 h post-infection (h p.i.), even though decreased cell viability was significant (as measured by trypan blue staining). Mock-infected cells proliferated while Asibi virus-infected hepatocytes had a 37.8% decrease in viability over the time course (Fig. 1b). In contrast, 17-D virus-infected cells had an 82.3% decrease in viability by 120 h p.i., (Fig. 1b). It is possible that 17-D virus replication is limited owing to overwhelming cell death, however, 17-D titres plateau when cell viability in both 17-D and Asibi virus-infected cells are essentially the same, (Fig. 1). These data demonstrate that both Asibi and 17-D viruses are capable of establishing productive infections. It also suggests that, in this closed hepatocyte environment, 17-D virus is more virulent than Asibi virus, as indicated by the excessive cell death.

17-D virus induces a prominent pro-inflammatory cytokine response in PH5CH8 hepatocytes

Supernatants from Asibi and 17-D virus-infected PH5CH8 hepatocytes were collected 1–120 h p.i. and evaluated for a small subset of cytokines: IL-1β, IL-4, IL-6, IL-8, IL-10 and TNF-α. These cytokines were selected since they have been suggested as being induced by YFV or having a role in disease progression (Belsher et al., 2007; Doblas et al., 2006; Hacker et al., 1998, 2001; Meulen et al., 2004; van der Beek et al., 2002; Verschuur et al., 2004). Hepatocytes infected with 17-D virus had a more pronounced response to infection by generally producing significantly ($P<0.05$) higher concentrations of pro- and anti-inflammatory cytokines than Asibi virus-infected hepatocytes during late-stage infections, (Fig. 2a, b). Significant differences mostly occurred between 72 and 120 h p.i., with exceptions to earlier time points (see full statistical descriptions in Supplementary Table S1, available in JGV Online). 17-D virus infection induced high concentrations (>20 pg ml$^{-1}$) of pro-inflammatory cytokines IL-6, IL-8 and TNF-α (Fig. 2a). IL-1β was produced in lower concentrations (<1.0 pg ml$^{-1}$) during late-stage infection when cells were undergoing rapid death. Hepatocytes infected with...
Asibi virus responded by producing pro-inflammatory cytokines that were significantly higher \((P<0.05)\) in concentration than mock-infected hepatocytes, but were markedly lower than in 17-D virus-infected hepatocytes. Anti-inflammatory cytokines IL-4 and IL-10 were also measured to determine whether regulatory mechanisms attributable to anti-inflammatory cytokines were functional in infected hepatocytes, and concentrations were found to be low in comparison to the pro-inflammatory cytokines detected (Fig. 2b). We observed that IL-4 release \((72–120\ h\ p.i.)\) was significantly higher \((P<0.05)\) from 17-D virus-infected hepatocytes when compared with Asibi virus-infected cells, (Fig. 2b). Asibi virus-infected hepatocytes typically produced levels of IL-4 that were not significantly different from mock infected cells \((except\ at\ 120\ h\ p.i.)\). IL-10 data had more variability between replicates and a statistically significant difference \((P<0.05)\) between YFV infections was observed only at 1 and 72 h p.i. However, with or without statistical significance, we found that hepatocytes infected with 17-D virus showed higher concentrations of IL-10 in media when compared with Asibi virus-infected hepatocytes, (Fig. 2b). Together, these cytokine data suggest that 17-D virus induces a strong pro-inflammatory cytokine response that may be necessary to signal professional immune cells.

Gene expression for IL-1\(\beta\), IL-4, IL-6, IL-8, IL-10 and the TNF superfamily generally correlated well with cytokine release data (Supplementary Fig. S1, available in JGV Online). Gene expression for pro-inflammatory cytokines IL-1\(\beta\), IL-6, IL-8 and the TNF superfamily was significantly upregulated \((P<0.05)\) following 17-D virus infection \((48–120\ h\ p.i.)\) with few exceptions and correlated with increased release of the corresponding cytokine, which sometimes occurred 24 h after increases in gene expression (Supplementary Fig. S1a). The same was true for anti-inflammatory cytokine IL-4 at 72–120 h p.i. (Supplementary Fig. S1b). IL-10, however, while showing significant differences in protein expression only at 1 and 96 h p.i., was significantly upregulated \((P<0.05)\) in Asibi virus-infected hepatocytes at 96–120 h p.i. (Supplementary Fig. S1b). See Supplementary Table S2 (available in JGV Online) for the statistical analysis for Supplementary Figs S1–S3 (available in JGV Online). These gene expression data may indicate that Asibi virus targets specific host-regulatory elements such as IL-10 expression.

### IL-6 pre-stimulation of PH5CH8 hepatocytes results in higher titres of both Asibi virus (wild-type) and 17-D (vaccine) virus

IL-6 is a major pro-inflammatory cytokine responsible for mediating fever and the acute-phase response (Rummel et al., 2006; Streetz et al., 2001). Vaccinees have been shown to have up to a 30 % increase in serum IL-6 5–6 days post-vaccination with 17-D virus (van der Beek et al., 2002). Since IL-6 is an important pro-inflammatory cytokine, we hypothesized that the presence of IL-6 prior to YFV infection would modulate the host response in hepatocytes. To investigate this we stimulated PH5CH8 hepatocytes with 5 ng IL-6 ml\(^{-1}\) prior to infection. Both Asibi and 17-D viruses retained their ability to replicate efficiently, and growth profiles were similar to infection of unstimulated hepatocytes (Fig. 3a). As in unstimulated cells, Asibi virus grew to significantly higher \((P<0.05)\) titres than 17-D virus. Interestingly, IL-6 pre-stimulation of hepatocytes appeared to induce more rapid replication as the growth profile of each virus was shifted earlier by approximately 24 h (Fig. 3c, d). This shift resulted in Asibi and 17-D virus reaching significantly higher \((P<0.05)\) titres at least 24 h before their unstimulated counterparts. However, after the early peak in titre, both Asibi and 17-D virus titres diminished during late-stage infection. Cell viability was also affected by IL-6 pre-stimulation as it decreased by 70 and 74.2 % in Asibi or 17-D virus-infected cells,

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**Fig. 1.** YFV growth kinetics in PH5CH8 hepatocytes and cell viability. Asibi (○) and 17-D (●) viruses replicate efficiently in PH5CH8 hepatocytes. (a) Asibi virus replicates to significantly higher titres \((*,\ P<0.05\ by\ Student’s\ t\text{-}test)\) when compared with 17-D virus. Data presented are means of biological and technical replicates in triplicate. (b) Cell viability was also assessed; mock-infected hepatocytes (solid line) grew over time whereas Asibi virus- (dotted line) and 17-D virus-infected (dashed line) hepatocytes both underwent considerable death (as measured by trypan blue staining).

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respectively (Fig. 3b). Mock-infected cells remained healthy over the 6 day time course. When compared with the cell viability of unstimulated hepatocytes, survivability for 17-D virus-infected hepatocytes improved, while Asibi virus-infected hepatocyte viability decreased.

IL-6 pre-stimulation of PH5CH8 hepatocytes enhances pro-inflammatory cytokine responses from both Asibi and 17-D virus infections

In addition to viral growth kinetics, we also evaluated the cytokine response to YFV infection following IL-6 pre-stimulation. IL-1β and TNF-α levels were significantly higher (P<0.05) for 17-D virus-infected hepatocytes at 72–120 h p.i. when compared with mock or Asibi virus-infected hepatocytes (Fig. 4a, b). IL-6 and IL-8 were also higher for 17-D virus-infected hepatocytes (Fig. 4a); however, the concentrations that were calculated to be significantly higher (P<0.05) for Asibi virus infection were at 24 and 72 h p.i. Anti-inflammatory cytokine IL-4 also remained significantly elevated (P<0.05) in 17-D virus-infected hepatocytes when compared with mock- or Asibi virus-infected cells (Fig. 4c). IL-10 concentrations, as in the unstimulated group, were found to have few significant differences between Asibi and 17-D virus-infected hepatocytes. However, we did observe an early and significant elevation in IL-10 concentration from Asibi virus-infected hepatocytes at 1 h p.i. (Fig. 4c), which is the opposite of the response seen in unstimulated cells (Fig. 2b).

IL-6 pre-stimulation of hepatocytes resulted in some critical differences when compared with the unstimulated group, as observed by using either Student’s t-test or a Mann–Whitney rank sums test. IL-6 pre-stimulation resulted in significantly higher levels (P<0.05) of IL-1β and IL-8 from Asibi virus infections at 72–120 h p.i. and 24–120 h p.i. (Fig. 4a), respectively, when compared with their unstimulated counterparts. These cytokines did not appear to be as greatly affected by the IL-6 pre-stimulation in 17-D virus-infected hepatocytes, as significant increases (P<0.05) were observed at 96–120 h p.i. (IL-1β) and 24–72 h p.i. (IL-8). IL-6 expression appeared to be affected by the pre-stimulation differently, depending on the infecting virus. Asibi virus infections resulted in significantly more (P<0.05) IL-6 release at 1–24 and 72–120 h p.i., while 17-D virus infection had significantly less IL-6 production compared with the unstimulated group at 24–48 h p.i. TNF-α concentrations were significantly lower after IL-6 pre-stimulation (P<0.05), regardless of the infecting virus, when compared with the unstimulated hepatocytes, (Fig. 4b). Anti-inflammatory cytokines IL-4 and IL-10 were both found in significantly higher
concentrations ($P<0.05$) from IL-6 pre-stimulated hepatocytes when compared with the unstimulated group, regardless of the infecting virus (Fig. 4c). IL-4 was significantly increased ($P<0.05$) after Asibi virus infection at 96–120 h p.i. and after 17-D virus infection at 72–120 h p.i. IL-10, however, was significantly increased for both infections at all time points (except for 17-D virus at 1 h p.i.), when compared with the unstimulated hepatocytes.

Gene-expression data for IL-6 pre-stimulated hepatocytes correlated less well with cytokine release data when compared with correlations seen in the unstimulated group. When compared with the unstimulated group, we found that these cytokines were divided into three groups: upregulated, downregulated and mixed. IL-1$\beta$ and IL-6 gene expression were found to mimic the cytokine release profiles where significantly higher ($P<0.05$) gene expression was seen in 17-D virus-infected hepatocytes (Supplementary Fig. S2a). These cytokines were also found to be significantly upregulated, regardless of the infecting virus, when compared with their unstimulated counterparts.

IL-8 gene expression was significantly upregulated ($P<0.05$) in Asibi virus-infected hepatocytes while TNF gene expression was observed to be significantly higher ($P<0.05$) following 17-D virus infection. When we compared IL-8 and TNF gene expression in IL-6 pre-stimulated hepatocytes with the unstimulated group, we found that hepatocytes infected with Asibi virus had upregulated expression of both genes, in contrast to hepatocytes infected with 17-D virus, in which both genes were downregulated (all reached statistical significance, $P<0.05$).

Gene expression of anti-inflammatory cytokines IL-4 and IL-10 (Supplementary Fig. S2c) was statistically equivalent between Asibi or 17-D virus infections in IL-6-stimulated groups. However, IL-4 and IL-10 gene expression were both statistically downregulated in IL-6 pre-stimulated hepatocytes, regardless of infecting virus, when compared with the unstimulated group.
TGF-β1 production and release is halted in YFV-infected PH5CH8 hepatocytes

TGF-β1 has previously been suggested as having a role in induction of apoptosis in hepatocytes during wild-type YFV infection (Quaresma et al., 2006). Here, we examined the release of TGF-β1 from mock-, 17-D virus- and Asibi virus-infected PH5CH8 hepatocytes. TGF-β1 is typically secreted from cells as a biologically inert protein needing activation by either proteolytic or non-proteolytic processes (Jenkins, 2008; Khalil, 1999). Early tests on our samples indicated that active TGF-β1 was not released from infected hepatocytes (data not shown). After activating the samples as recommended by the assay manufacturer (Millipore), we detected TGF-β1 and found that mock-infected hepatocytes released significantly more TGF-β1 over time than either of the YFV-infected groups (P < 0.05) (Fig. 5a, b). IL-6 pre-stimulation of hepatocytes resulted in more TGF-β1 being released (Fig. 5b), but concentrations remained well below those observed for mock-infected samples.

Since TGF-β1 cytokine release was diminished in YFV-infected hepatocytes relative to mock-infected cells, we conducted gene expression assays to determine whether there was up- or downregulation of the TGF-β1 gene. We found that TGF-β1 gene expression remained relatively consistent over time in both unstimulated and IL-6 pre-stimulated hepatocytes following YFV infection (Supplementary Fig. S3).

These data suggest that YFV infection does not affect TGF-β1 gene expression, but may inhibit release of the cytokine in either its active or inactive form.

To determine whether hepatocytes were retaining TGF-β1, we conducted confocal microscopy studies at 1 and 96 h p.i. We found that hepatocytes had minimal staining for TGF-β1 at 1 or 96 h p.i. in mock-, Asibi virus- or 17-D virus-infected groups (data not shown). Consistent gene expression, diminished release of protein into the media and no evidence of retention of protein in the cytoplasm suggests that, in hepatocytes, infection by 17-D or Asibi virus may inhibit translation of TGF-β1 mRNA. Additional studies are needed to characterize the role of TGF-β1 during YFV infection, but our study suggests that release of TGF-β1 from hepatocytes is not a major contributor to the host response or liver pathology. These data lead us to believe that TGF-β1 in the liver is released from hepatic cells other than hepatocytes.

DISCUSSION

The overall objective of our work with YFV is to understand host responses that are associated with the development of disease and virus-induced pathogenesis. The studies reported here compared the hepatocyte response to infection with wild-type (Asibi) and vaccine
(17-D) YFV strains. We have previously reported results from vascular endothelial cells and Kupffer cells (Khaiboullina et al., 2005; Woodson et al., 2011), which indicate that wild-type and vaccine strains of YFV stimulate markedly different host responses. We have also shown that IL-6 appears to be a significant component of the host response (Khaiboullina et al., 2005). Here, we turned our focus to the principal cellular component of the liver: hepatocytes. For these studies we used the non-neoplastic PH5CH8 hepatocyte line rather than primary cells owing to their availability and for consistency between experiments.

Initial studies to demonstrate viral infection and replication competence found that PH5CH8 hepatocytes supported replication of both Asibi and 17-D virus. Asibi virus replicated more efficiently than 17-D virus in both unstimulated and IL-6 pre-stimulated hepatocytes, reaching peak titres approximately 2 logs higher. IL-6 pre-stimulation of hepatocytes appeared to increase the kinetics of virus propagation, yet decreased long-term virus production when compared with unstimulated hepatocytes.

Evaluation of the host cytokine response to YFV infection found clear and distinct differences between hepatocytes infected with wild-type or vaccine strains. In unstimulated PH5CH8 hepatocytes, 17-D virus infection resulted in the release of IL-1β, IL-6, IL-8, TNF-α and IL-4 at concentrations that were significantly higher than in either mock- or Asibi virus-infected hepatocytes. These data indicate that 17-D virus infection induces a strong pro-inflammatory response in hepatocytes, with potential regulatory control driven through IL-4, but not IL-10 as we saw with Kupffer cells (Woodson et al., 2011). The elevated expression of IL-4 indicates that hepatocytes may stimulate alternative activation (type M2a) (Martinez et al., 2008, 2009) of resident (Kupffer cells) or circulating macrophages following 17-D virus infection. The lack of this response in hepatocytes infected with Asibi virus suggests that either the pro-inflammatory and IL-4 response is inhibited or the intracellular pathways regulating these responses are not activated.

IL-6 pre-stimulation of hepatocytes enhanced IL-1β, IL-8, IL-4 and IL-10 production following infection with either virus, but especially Asibi virus (greatest cytokine increases in this group). However, IL-6 pre-stimulation of hepatocytes caused TNF-α to be significantly decreased after both infections, and IL-6 production was differentially affected depending on the infecting virus when compared with the unstimulated hepatocytes. These data suggest that early IL-6 increases may potentiate the inflammatory response to wild-type virus infection in hepatocytes and raises questions regarding potential positive- or negative-feedback control of hepatocytes following YFV infection. Pre-stimulation of hepatocytes with IL-6 appears to ameliorate inhibitory properties controlled by Asibi virus as the host response in IL-6 pre-stimulated hepatocytes is similar in both 17-D and Asibi virus infections. Proposing mechanisms that regulate this response is premature, as further studies are warranted.

Appropriate pro- and anti-inflammatory responses are critical for the control of YFV infection. Studies on serum samples collected from clinically ill patients during YF outbreaks correlated high levels of IL-6 and IL-8 with poor outcomes (ter Meulen et al., 2004). A similar correlation between IL-8 and disease severity has been observed in dengue virus infections (Raghupathy et al., 1998). Interestingly, dengue virus has also been shown to induce IL-8 release from hepatocytes (Suksanpaisan et al., 2007). While pro-inflammatory responses are critical for stimulating a response to eliminate the virus or infected cells, the anti-inflammatory mechanisms are vital for regulating pro-inflammatory cytokine production to prevent an excessive response. In our studies, we found that 17-D virus...
infection of hepatocytes induced a strong pro-inflammatory response and counter response from IL-4, indicating an appropriate inflammatory response. Infection of unstimulated cells with Asibi virus stimulated neither a pro-inflammatory response nor a significant anti-inflammatory response. In some fatal cases of vaccine-associated viscerotropic disease there are reports of relatively high levels of the anti-inflammatory cytokines IL-4 and IL-10 (Belsher et al., 2007; Doblas et al., 2006). These data suggest that poor regulation of both the pro- and anti-inflammatory responses may contribute to the development of severe disease and also provide some evidence that wild-type YFV may be able to block or reduce these responses.

IL-6 is an important mediator of fever development and a key regulator of the acute phase response (APR). In principal, the APR should function to limit tissue damage associated with the immune system following infection (Suffredini et al., 1999). In the case of 17-D infection, induction of a strong IL-6 response from hepatocytes would function to limit the progression of the infection and, perhaps, also limit disease severity. Abnormalities in IL-6 signalling or release in Asibi virus-infected hepatocytes may alter the APR and contribute to the coagulation abnormalities that are seen during severe YF.

TGF-β1 has previously been implicated in YFV-induced liver pathology (Quaresma et al., 2006) and we expected to find upregulation of TGF-β1 in YFV-infected hepatocytes. However, we found that hepatocytes released less TGF-β1 after infection with either Asibi or 17-D viruses when compared to control hepatocytes, even though gene expression was consistent among all three groups. We also found that the inability to detect TGF-β1 in cell-culture supernatants was not because of intracellular retention of TGF-β1 by hepatocytes. These data indicate that, in hepatocytes, TGF-β1 production is suppressed at the level of translation by both Asibi and 17-D viruses. TGF-β1 has been suggested to induce apoptosis in hepatocytes during YFV infection (Cain & Freathy, 2001; Quaresma et al., 2006), however, our data do not support this hypothesis. During natural infection of the liver, it is possible that TGF-β1 may be produced by hepatic vascular endothelial cells or Kupffer cells, which could stimulate hepatocyte apoptosis through paracrine signalling. Interestingly, TGF-β1 activation in the liver can disrupt extracellular matrix synthesis and result in fibrosis (Szabo et al., 2007). The convalescent YF liver shows no evidence of fibrosis and returns to normal histology (Klotz & Belt, 1930; Monath & Barrett, 2003). Clearly the role of TGF-β1 in the YF liver needs further investigation, but in hepatocytes it does not appear to play a major role in pathogenesis.

Yellow fever vaccination carries a risk of serious adverse events, one of which is development of disease similar in presentation to wild-type virus infection. In a normal system, 17-D virus appears to stimulate an appropriate immune response whereas wild-type virus may be able to limit the inflammatory response and thus escape professional immune-cell detection. In the case of vaccine-associated viscerotropic disease, we suggest that a defect in innate immune sensing or an abnormality with professional immune-cell activation in the liver results in progressive infection that presents as wild-type infection. Future studies are needed to identify specific cellular response mechanisms associated with the development of severe disease following vaccination.

In summary, PH5CH8 hepatocytes support infection and replication of both Asibi and 17-D viruses. Even though 17-D virus replicated to lower titres than Asibi virus, infection of unstimulated hepatocytes resulted in decreased cell viability and elevated expression of pro-inflammatory cytokines and IL-4. In this system, 17-D virus appears to be more pathogenic than wild-type, Asibi, virus. However, less efficient replication by 17-D virus suggests restricted virus dissemination. In addition, induction of a strong pro-inflammatory response in hepatocytes by 17-D virus may be an effective signal in vivo to Kupffer cells, T-cells and infiltrating macrophages, alerting them to the pathogen. The restricted response seen in unstimulated hepatocytes infected with Asibi virus, coupled with the high replication titre, may indicate an effective means of virus dissemination without the appropriate antiviral host response.

**METHODS**

**Cells.** PH5CH8 hepatocytes are a simian virus 40 large T antigen-immortalized non-neoplastic human hepatocyte cell line that was provided by Dr Nobuyuki Kato (Okayama University, Japan). PH5CH8 hepatocytes were selected because they have been previously described as having intact antiviral pathways, such as Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene 1 (RIG-I)/melanoma differentiation associated gene-5 (MDA5), and are more similar to primary human hepatocytes than typical immortalized hepatocytes (Preiss et al., 2008). PH5CH8 hepatocytes were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g D-glucose and L-glutamine l-1 (Invitrogen), 10 % heat inactivated FBS, 1 % of 100 x non-essential amino acids (Sigma) and 1 mM sodium pyruvate (Sigma). PH5CH8 hepatocytes were plated at cell density of 10⁵ cells per well in 24-well cell-culture plates and allowed to adhere and grow for 24 h. Cell density was chosen to mimic the confluency of cells in human liver. Prior to viral infection, cell density was determined (by using trypan blue) in one representative well and cells were checked to ensure they had not become overconfluent.

Vero-E6 cells (ATCC, CRL1586) were used for viral-stock growth and immuno-focus forming assays and were maintained in 1 × modified Eagle’s medium supplemented with Earle’s balanced salts, 2 mM L-glutamine (Invitrogen), 1 % of 100 x non-essential amino acids 1 mM sodium pyruvate (Sigma) and 10 % bovine growth serum (BGS; Hyclone). Media used for viral growth stock contained 2 % BGS.

**IL-6 pre-stimulation of PH5CH8 hepatocytes.** PH5CH8 hepatocytes plated for use in this study were divided into two equal groups: unstimulated and IL-6 pre-stimulated. PH5CH8 hepatocytes were treated with 5 ng recombinant human IL-6 ml⁻¹ (R&D Systems) for 5 days to derive the IL-6 pre-stimulated group. The unstimulated PH5CH8 hepatocyte group did not receive any exogenous IL-6, just maintenance media as control.
Viruses. Two YFV strains, wild-type and vaccine, were used in this study. Dr Robert Tesh [University of Texas Medical Branch, Galveston, Texas, USA and World Reference Collection for Emerging Viruses and Arboviruses (WRCEVA)] provided the wild-type strain, Asibi virus. Dr Alan Barrett (University of Texas Medical Branch, Galveston, Texas, USA) provided the vaccine strain, 17-D-204, which was derived from a 17-D infectious clone (Rice et al., 1989). Vero-E6 cells were used to grow both virus working stocks. Virus working stocks were harvested when cytopathic effects were first visible.

Immu-no-focus forming assays. Immuno-focus forming assays were used to determine the titres of the working viral stocks and of each sample collected (all assays were performed in triplicate). The immuno-focus forming protocol was previously described by Woodson et al. (2011).

Viral growth kinetics. PH5CH8 hepatocytes were infected with either Asibi or 17-D virus at an m.o.i. of 4.0 diluted in complete medium containing 2% BGS (Hyclone). Control hepatocytes were mock-infected with the same media used for viral dilutions. Unstimulated and IL-6 pre-stimulated experiments were performed consecutively with equivalent cell density and passage history; three replicate time courses were performed. Hepatocytes were infected with 100 μl of viral suspension for 1 h at 37 °C with occasional rocking, washed three times in PBS (pH 7.2) and fresh media added (1 ml). Supernatants and cell lysates (in lysis buffer containing proteinase K; Panomics) were collected at 1–120 h p.i. All samples were stored at −80 °C until analysed.

Cytokine determination. Multiplex magnetic bead-based assays available from Bio-Rad and Millipore were used to detect and measure cytokines IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α and TGF-β1. Samples were tested at each time point were removed. Samples tested were not diluted and each was tested in triplicate. Samples were tested by using a Bio-Rad Bio-plex 200 machine set for reading at high sensitivity, i.e. the machine was set to detect low concentrations. The manufacturer’s instructions were used for all assays.

Gene expression determination. A multiplexed bead-based assay was also used to detect and measure mRNA transcripts for the above cytokines, which was purchased from Panomics (now Affymetrix). Panomics designed the probes for the targets based upon GenBank [National Center for Biotechnology Information (NCBI)] sequence data. Cellular lysates were prepared as per the manufacturer’s instructions and were tested in triplicate. All samples were tested at high sensitivity on a Bio-Rad Bio-Plex machine (Bio-Rad). The gene expression data were normalized against two housekeeping genes: GAPDH and the β-actin gene. Fold changes in expression were calculated for the infected samples from mock-infected control values. All mock-infected control data are equal to a value of 1, which is not displayed in Supplementary Figs S1–S3.

Confocal microscopy studies. PH5CH8 hepatocytes were grown on glass coverslips in 24-well cell culture dishes for 1 day before infection with Asibi or 17-D virus at an m.o.i. of 4.0, or mock-infected with complete medium. At 96 h post-infection, the hepatocytes were fixed with 10% formalin and permeabilized with PBS containing 0.2% Triton X-100 for 5 min at room temperature. Coverslips were washed with PBS three times, incubated with Fc receptor block (Innovex) for 30 min, washed with PBS three times and primary antibodies were added. The primary antibodies used were hyper-immune mouse ascitic fluid against YFV-Asibi virus (WRCEVA), rabbit pan-specific TGF-β antibody (R&D Systems), and for isotype controls normal mouse IgG and rabbit IgG (R&D Systems) diluted in PBS containing 2% goat serum (Sigma). Primary antibody was incubated on the coverslips for 30 min at room temperature with rocking. Coverslips were washed with PBS three times before the addition of secondary antibodies. The secondary antibodies used were goat anti-mouse IgG–Alexa Fluor 594 (H+L) and goat anti-rabbit IgG–Alexa Fluor 488 (Invitrogen) diluted in PBS containing 2% goat serum (Sigma). Secondary antibodies were incubated in the dark for 10 min and washed three times with PBS. Coverslips were mounted on glass slides with SlowFade Gold DAPI (Invitrogen) and viewed on an Olympus Fluoview 1000MPE system with an upright BX61 microscope (Galveston National Laboratory, Galveston, Texas, USA).

Statistical analyses. All experiments and assays reported here were performed in triplicate (biological and technical) to achieve maximum statistical significance. Student’s t-tests were used to assess significant differences (P<0.05) for viral replication and some cytokine production and gene expression, as indicated in the figures by an asterisk (*). Not all cytokine and gene-expression datasets passed tests for normality and/or equal variance. For those datasets that failed, the Mann–Whitney rank sums test was used to assess significance (P<0.05). All data were considered significant if P<0.05 (95% confidence interval) was achieved by using these methods.

REFERENCES


Hepatocyte response to Asibi and 17-D YFV infection


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