Virology 412 (2011) 188–195

Contents lists available at ScienceDirect

Virology

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

Differential cytokine responses from primary human Kupffer cells following infection with wild-type or vaccine strain yellow fever virus

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ARTICLE INFO

Article history:
Received 27 October 2010
Returned to author for revision
8 December 2010
Accepted 7 January 2011
Available online 1 February 2011

Keywords:
Kupffer cell
Yellow fever virus
IL-8
IL-10
RANTES/CCL5
TNF-α

ABSTRACT

Wild-type yellow fever virus (YFV) infections result in a hepatotropic disease which is often fatal, while vaccination with the live-attenuated 17-D strain results in productive infection yet is well-tolerated with few adverse events. Kupffer cells (KCs) are resident liver macrophages that have a significant role in pathogen detection, clearance and immune signaling. Although KCs appear to be an important component of YF disease, their role has been under-studied. This study examined cytokine responses in KCs following infection with either wild-type or vaccine strains of YFV. Results indicate that KCs support replication of both wild-type and vaccine strains, yet wild-type YFV induced a prominent and prolonged pro-inflammatory cytokine response (IL-8, TNF-α and RANTES/CCL5) with little control by a major anti-inflammatory cytokine (IL-10). This response was significantly reduced in vaccine strain infections. These data suggest that a differentially regulated infection in KCs may play a critical role in development of disease.

Introduction

Yellow fever virus (YFV) (family *Flaviviridae,* genus *Flavivirus*) is the causative agent of yellow fever (YF), an often fatal viral hemorrhagic fever. YFV infection carries a 20–50% mortality rate and it is estimated that approximately 30,000 deaths occur annually due to this disease (World Health Organization). The 17-D virus, is the live-attenuated vaccine strain of YFV that is typically very well-received by the vaccinee and confers protection for at least 10 years following a single inoculation. 17-D virus has been used for more than 70 years with over 500 million doses administered and with relatively few serious adverse events (Monath et al., 2010). However, as techniques for detecting and reporting serious adverse events improve, an increasing number of YF vaccine associated serious adverse events (YFV-SAEs) have been reported.

YFV is highly hepatotropic, sometimes involving as much as 80% of hepatocytes in infected humans (Klotz and Belt, 1930). YFV infection results in five distinct liver pathologies: lesions of the midzone, steatosis, severe inflammation with disproportionate inflammatory cell infiltrate, eosinophilic degeneration of hepatocytes and Kupffer cells (Councilman bodies), and upon resolution of infection, a complete return to normal histology (Monath and Barrett, 2003). Even though these pathologies have been well characterized little is known about mechanisms associated with their development.

Kupffer cells (KCs) are resident liver macrophages responsible for detecting pathogens and activating local immune responses. KCs' role in disease development appears to be critical as evidence suggests they are the first cells to become infected when YFV enters the liver (Bearcroft, 1957; Klotz and Belt, 1930; Tigertt et al., 1960). Little is known about the pathogenesis of YFV in KCs; however, because of their involvement in early infection and potential immune activation, we hypothesize that these cells play a significant role in YFV pathogenesis. This study compared viral growth kinetics and cytokine responses in KCs following infection with either wild-type (Asibi virus) or vaccine (17-D virus) strains of YFV. Wild-type YFV was found to replicate more efficiently and to induce a more profound pro-inflammatory response in KCs compared to infection with the 17-D vaccine strain. These data suggest that poor regulation of infection by KCs may be a critical component for development of severe disease.

Results

Kupffer cells increase cell surface markers CD14, CD86 and HLA-DR after IL-6 stimulation

Kupffer cells are professional immune cells within the liver and their activation by cytokine signals and pathogenic antigens are critical for inducing protective responses. Kupffer cells, as other macrophages, can become activated by pathogen-associated

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0042-6822/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2011.01.012

membrane proteins (PAMPs, such as LPS) via toll-like receptors or primed by exogenous cytokines (Bilzer et al., 2006; ten Hagen et al., 1998). Some studies have reported early and prominent serum increases of IL-6 in YFV vaccinees as well as from other cells types, such as endothelial cells, during wild-type virus infection (Khaiboullina et al., 2005; van der Beek et al., 2002; Verschuur et al., 2004). To determine if early exogenous increases in IL-6 would modulate activation and subsequent response of KCs to infection with Asibi and 17-D viruses, unstimulated and IL-6 pre-stimulated primary KCs were used in this study. Prior to viral infection, unstimulated and IL-6 pre-stimulated KCs were tested by flow cytometry for cell surface markers CD14, CD86 and HLA-DR (common macrophage markers) to assess the purity of each population and activation state after IL-6 stimulation. Using these markers we were able to conclude that both populations were >90% pure (data not shown). Compared to unstimulated KCs, all three cell surface markers were increased after IL-6 stimulation for 5 days. CD14 and HLA-DR increased by 2.9- and 2.4-fold, respectively, while CD86 increased to a lesser extent at 1.6-fold (data not shown). Kupffer cell surface marker up-regulation suggests these cells become activated in response to exogenous IL-6 stimulation.

Asibi and 17-D viruses replicate efficiently in Kupffer cells

Kupffer cells appear to be the first cells infected upon wild-type YFV invasion of the liver; however, no data are available to determine if the vaccine strain, 17-D virus, is capable of infecting or replicating efficiently in these cells. We wanted to determine if KCs support infection and replication of both Asibi virus (wild-type) and 17-D virus (vaccine strain) and if replication efficiency was modulated by IL-6 pre-stimulation of KCs. Fig. 1A demonstrates that Asibi virus replicates to significantly ($p<0.05$) higher titers than 17-D virus (except at 24 h post-infection) in unstimulated KCs and is detectable through 144 h post-infection. IL-6 pre-stimulation improved the replication efficiency of Asibi virus, but not 17-D virus (Fig. 1B), resulting in significantly higher titers of Asibi virus. In contrast, replication of 17-D virus was not affected by IL-6 pre-stimulation of KCs (Fig. 1 A and B). IL-6 pre-stimulated KCs also had sustained high titers of Asibi virus, lasting through 120 h post-infection, whereas unstimulated KCs only had a short-lived single peak titer.

Cell viability was also affected by YFV infection where unstimulated KCs infected with either Asibi virus and 17-D virus had 32% and 47% viability at 144 h post-infection, respectively. IL-6 pre-stimulation of KCs improved viability of Asibi virus infected cells with 52% viable at 144 h post-infection. In contrast, IL-6 pre-stimulation did not significantly enhance cell survival (49% live at 144 h post-infection) after 17-D virus infection.

Asibi virus infection elicits strong pro- and anti-inflammatory responses from unstimulated Kupffer cells

One of the most important activities of KCs is to delineate between self and pathogenic antigens. An example of KC regulation of liver homeostasis is through release of anti- and pro-inflammatory cytokines. For this study, we chose to investigate a small subset of cytokines, IL-8, IL-10, RANTES/CCL5 and TNF-α, which previous publications suggest may be important in the development or progression of disease during YFV infections (Bae et al., 2008; Belsher et al., 2007; Doblas et al., 2006; Gaucher et al., 2008; Hacker et al., 1998; Khaiboullina et al., 2005; ter Meulen et al., 2004; van der Beek et al., 2002). Cytokine responses were measured from unstimulated KCs over a 6-day period following mock infection or infection with either Asibi virus or 17-D virus.

In general, Asibi virus infection of unstimulated KCs resulted in overall higher cytokine expression, at both the transcriptional and translational level when compared to either mock or 17-D virus infected cells (Fig. 2). IL-8 cytokine expression was up-regulated early during infection (48 h post-infection), while TNF-α, RANTES/CCL5 and IL-10 were all up-regulated later (96 h post-infection) (Fig. 2A–D). Gene expression for IL-8, TNF, RANTES/CCL5 and IL-10 corresponded reasonably well with few exceptions to cytokine expression (Fig. 2A–D). Gene expression for TNF was up-regulated extensively in the 17-D virus infected group early during infection without an increase in TNF-α release. However, this gene encodes for the whole TNF superfamily and may not directly represent gene expression specific to TNF-α production.

IL-6 pre-stimulation of Kupffer cells modulates pro- and anti-inflammatory cytokine responses to infection with Asibi virus and 17-D virus

IL-6 pre-stimulated KCs were also tested for the expression profiles of IL-8, TNF-α, RANTES/CCL5 and IL-10 post-infection with Asibi virus, 17-D virus or mock (control). IL-8, TNF-α and RANTES/CCL5 remained significantly higher ($p<0.05$) from Asibi virus infected

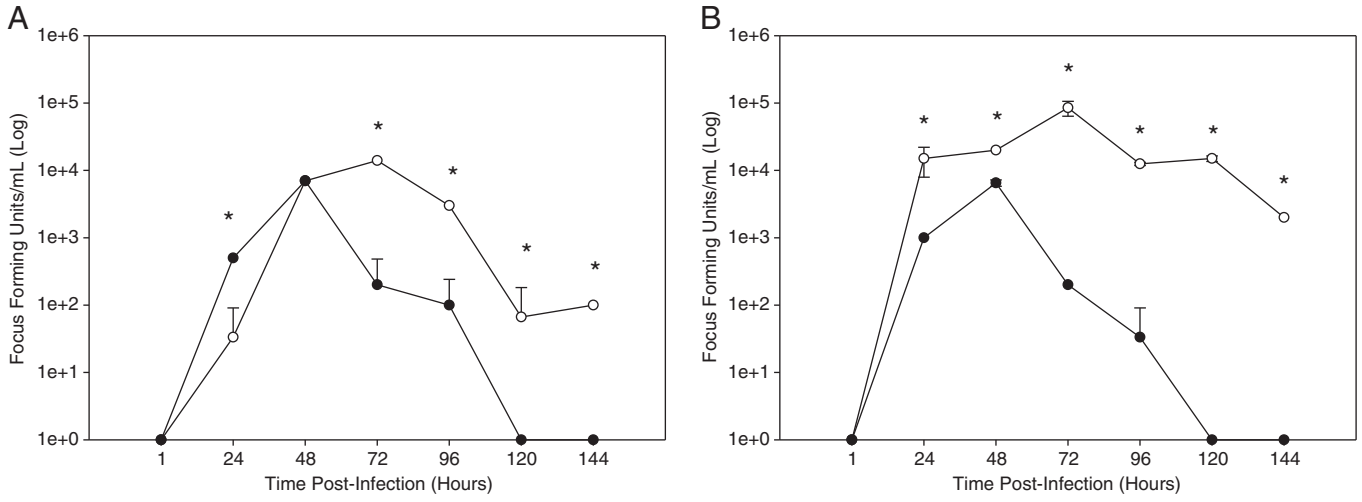
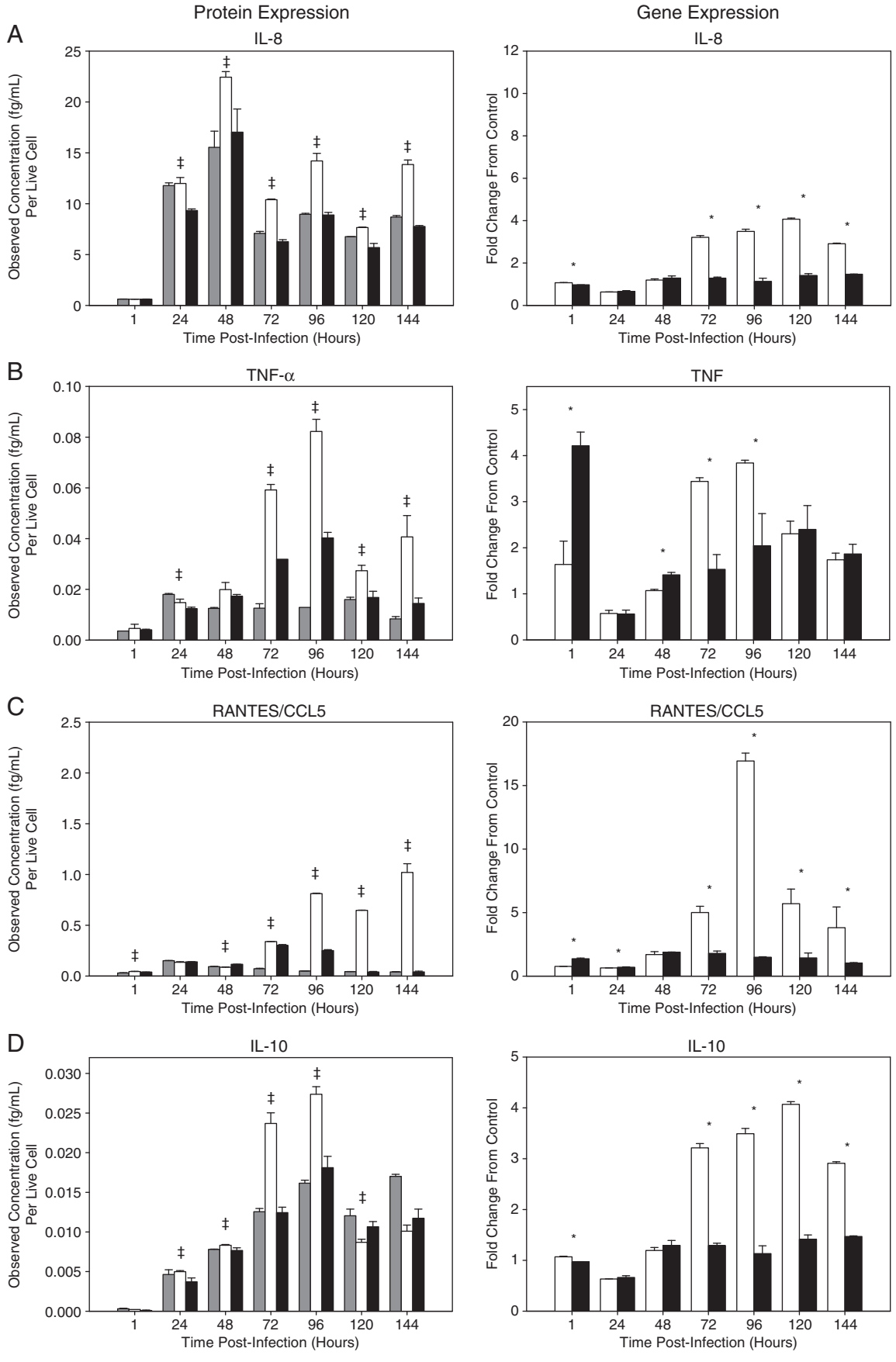


Fig. 1. Wild-type and vaccine strain YFV replication kinetics in unstimulated and IL-6 pre-stimulated Kupffer cells. Asibi virus and 17-D virus replication was evaluated in unstimulated (A) and IL-6 pre-stimulated (B) Kupffer cells by immuno-focus assay. Asibi virus (○) replicates to significantly (* indicates $p<0.05$) higher titers than 17-D virus (●) in both KC groups. IL-6 pre-stimulation of KCs does not appear to effect 17-D virus replication. Supernatants for each time point were harvested from separate wells.


Protein Expression
Gene Expression
A
IL-8
IL-8
Observed Concentration (fg/mL) Per Live Cell
Fold Change From Control
Time Post-Infection (Hours)
B
TNF-α
TNF
C
RANTES/CCL5
RANTES/CCL5
D
IL-10
IL-10
1
24
48
72
96
120
144

KCs in comparison to 17-D infected cells (Fig. 3A–C). We detected a significantly ($p<0.05$) up-regulated expression level of TNF-α and RANTES/CCL5 in the IL-6 pre-stimulated KCs after infection, regardless of the infecting virus. In contrast to unstimulated cells, a temporal difference in release of IL-8 was observed. At early time points post-infection release of IL-8 was down-regulated, while at later stages of the infection an up-regulation was detectable. We also observed that IL-10 cytokine release was significantly down-regulated from both YFV infections (Fig. 3D), but overall IL-10 concentrations were higher following 17-D virus infection. Gene expression of IL-8, TNF-α, RANTES/CCL5 and IL-10 did not appear to be greatly up- or down-regulated in IL-6 pre-stimulated KCs when compared to unstimulated KCs. This result may indicate that IL-6 pre-stimulation of KCs (and subsequent activation) results in more efficient translation or release of these cytokines.

Discussion

KCs are one of the three principal cell types within the liver along with hepatocytes and endothelial cells. As a specialized macrophage the principal roles of Kupffer cells are phagocytosis, antigen presentation and recruitment of additional immune cells to sites of inflammation in the liver. KCs are found lining the sinusoids and have direct contact with hepatocytes. Previous studies indicate that KCs are the first to become infected during YFV infection, and because of their function, may be an important modulator of the immune response in the liver (Bearcroft, 1957; Klotz and Belt, 1930; Smetana, 1962; Tigertt et al., 1960). The response of KCs to infection is vital towards maintaining overall liver health and any inhibition or exacerbation of their response by a pathogen could lead to more severe infection and, given the association of the liver with the vascular system via the hepatic vein, potentially more efficient dissemination of the pathogen.

In this study we wanted to investigate whether the response of KCs to infection with wild-type or vaccine strains of YFV was significantly different to determine if KCs might play a key role in the development of disease. Further, we wanted to evaluate whether the infection of KCs could be exacerbated by IL-6. Previous works have indicated that wild-type infection and vaccination induced a significant release of IL-6 in patients (Belsher et al., 2007; Doblas et al., 2006; ter Meulen et al., 2004; van der Beek et al., 2002; Verschuur et al., 2004). Our results showed that primary KCs allow for more efficient replication of wild-type YFV than the vaccine strain and that this difference is amplified in the presence of IL-6. Further, we have shown that wild-type YFV infection of KCs stimulates a significantly more pronounced inflammatory cytokine response than infection with the vaccine strain. These data demonstrate that KCs may be unable to regulate infection by wild-type YFV and the subsequent pro-inflammatory response may play a significant role in the development of severe disease.

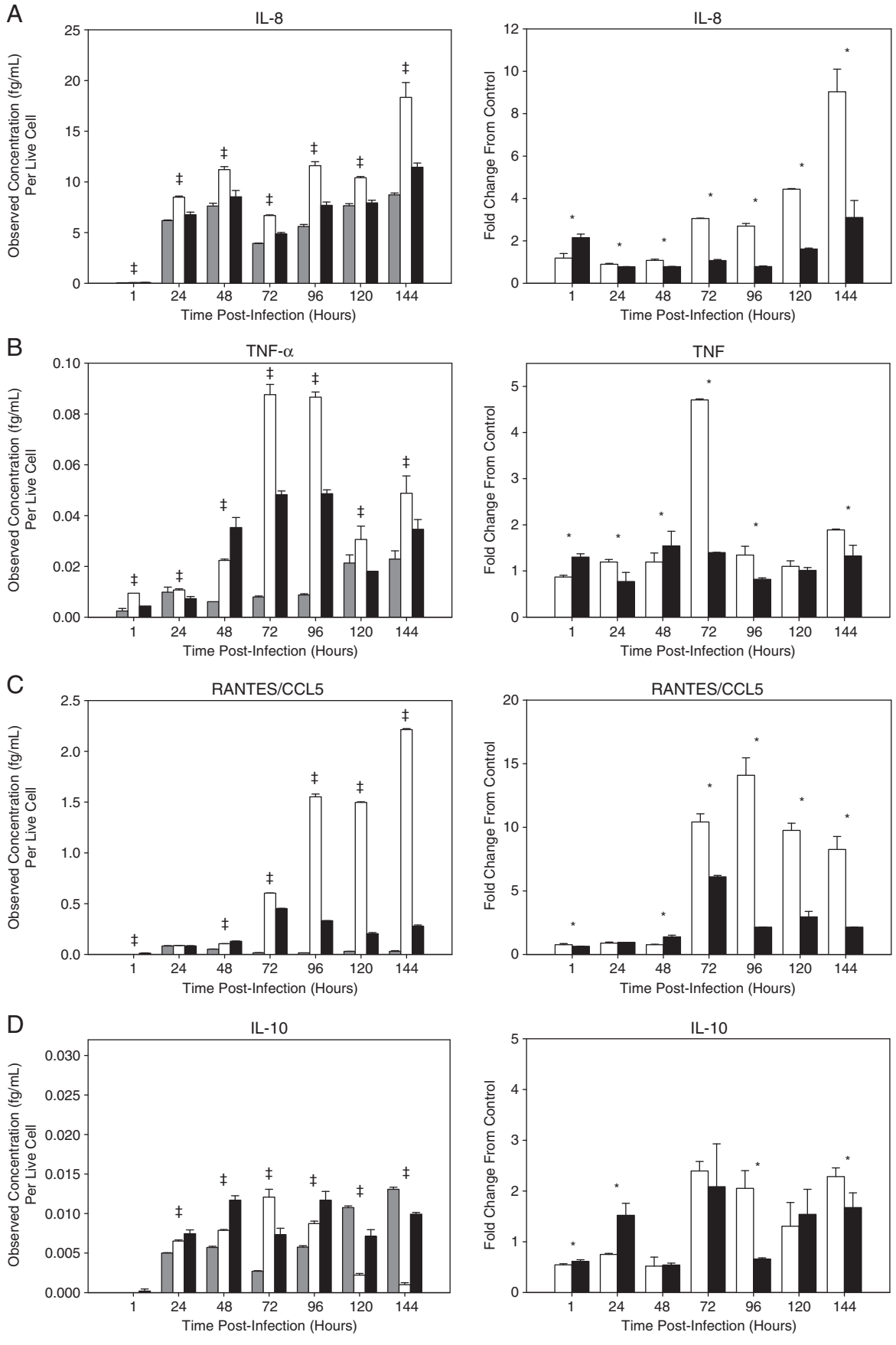
The cytokine expression profile of three out of four key inflammatory mediators was essentially the same in cells that were either naïve or pre-stimulated with IL-6 prior to infection. However, differential regulation was demonstrated for IL-10 between naïve and IL-6 pre-stimulated cells following infection with wild-type YFV. The primary function of IL-10 is anti-inflammatory in nature and has been shown to be an important signaling molecule between natural killer cells and KCs (Tu et al., 2008; Zhang et al., 2010). Activation of TLR3 by dsRNA (such as YFV replication intermediates) stimulates down-regulation of IL-10 and up-regulation of pro-inflammatory cytokines (Tu et al., 2008). We did not observe this "normal" response in KCs infected with Asibi virus. IL-10 transcriptional and translational expression levels were significantly higher in KCs infected with wild-type Asibi virus than in naïve cells infected with the vaccine strain 17-D, suggesting an active effort in regulating the host response. High gene expression of IL-10 has also been observed in the livers of Jimenez strain (hamster adapted wild-type YFV) infected Syrian golden hamsters (Li et al., 2008). We also found that pre-stimulation with IL-6 mitigated the production and release of IL-10 at the level of transcription in Asibi infected cells, indicating that this virus, in the presence of IL-6, could effectively block the regulatory role of IL-10. Such a situation could give rise to an unrestricted inflammatory response resulting in the observed characteristic development of cellular apoptosis in the liver of YF patients. The activity of IL-10 in regulating the host immune response may be a key element in the differential response between infections with wild-type or vaccine strains of YFV.

Kupffer cells, like circulating macrophages, can also produce a number of pro-inflammatory cytokines including IL-8, TNF-α and RANTES/CCL5. We found that all three of these cytokines were significantly up-regulated in Asibi virus infected KCs regardless of their IL-6 pre-stimulation status. Release of all three cytokines was generally increased in 17-D infected cells, but not to the magnitude observed for cells infected with wild-type Asibi virus. Gene expression data largely mimicked protein release indicating that cytokine production was regulated predominately at the transcriptional level. Analysis of IL-6 mRNA indicated that Asibi virus infection also up-regulated expression of IL-6 to a level greater than in 17-D infection (data not shown), perhaps indicating the potential for deleterious autocrine signaling.

The up-regulation of IL-8, TNF-α and RANTES/CCL5 in KCs indicates the activation of a pro-inflammatory response following infection with YFV. TNF-α is well known to be associated with activation of NF-κB which regulates IL-6 and IL-8 expression (Roebuck, 1999; Schutze et al., 1995; Vanden Berghe et al., 2000). TNF-α also has pro-apoptotic properties (Tacke et al., 2009). Subsequently, very high concentrations of TNF-α from KCs could be cytotoxic to surrounding hepatocytes, whereas lower levels can induce a pro-proliferative state for these cells (Dong et al., 2007; Smedsrod et al., 2009). Since Asibi virus infection induced significantly higher concentrations of TNF-α than 17-D virus, this may indicate that part of the necrosis and apoptosis seen in hepatocytes during late stage disease is due to an elevated TNF-α level within the liver. TNF-α expression was also observed to be elevated during early and late stage with a decrease at mid-stage disease in livers from the YFV Syrian golden hamster model (Li et al., 2008). Since 17-D virus is attenuated and does not appear to induce a strong pro-inflammatory response, it is possible that the amount of TNF-α produced is sufficient to control viral replication.

Expression of IL-8 was also elevated in wild-type Asibi virus infected KCs relative to both mock and 17-D virus infected cells. Like IL-6, IL-8 is a major component of the pro-inflammatory response whose expression is also regulated through NF-κB (Roebuck, 1999). IL-8 is readily produced from KCs, but can also result in a prolonged state of inflammation (Szabo et al., 2007). IL-8 can activate neutrophils and cause them to migrate to areas of inflammation (Harada et al., 1994). The presence of high levels of IL-8 suggests that wild-type, and to a lesser extent vaccine strain, YFV infection would induce significant neutrophil infiltration into the liver, however this has not been observed in post-mortem examinations (Klotz and Belt,

Fig. 2. Cytokine and gene expression from YFV infected unstimulated Kupffer cells. Unstimulated Kupffer cells were either mock (gray bars), Asibi virus (white bars) or 17-D virus (black bars) infected and subsequent cytokine (left side) and cytokine gene expression (right side) were measured. Pro-inflammatory and anti-inflammatory cytokines IL-8 (A), TNF-α (B), RANTES/CCL5 (C) and IL-10 (D) all had similar profiles of release that corresponded reasonably well with gene expression. Asibi virus infected KCs had significantly higher concentrations of cytokines (‡, centered over time point, indicates $p<0.05$) and gene expression (*, centered over time point, indicates $p<0.05$) when compared to 17-D virus infected cells. All data presented here is the average of triplicate measurements. Gene expression data is presented as fold change versus control.


A
IL-8
Observed Concentration (fg/mL) Per Live Cell
Time Post-Infection (Hours)
IL-8
Fold Change From Control
Time Post-Infection (Hours)
B
TNF-α
Observed Concentration (fg/mL) Per Live Cell
Time Post-Infection (Hours)
TNF
Fold Change From Control
Time Post-Infection (Hours)
C
RANTES/CCL5
Observed Concentration (fg/mL) Per Live Cell
Time Post-Infection (Hours)
RANTES/CCL5
Fold Change From Control
Time Post-Infection (Hours)
D
IL-10
Observed Concentration (fg/mL) Per Live Cell
Time Post-Infection (Hours)
IL-10
Fold Change From Control
Time Post-Infection (Hours)

1930; Smetana, 1962; Tigertt et al., 1960). The mechanism behind the disproportionate levels of IL-8 and neutrophil migration remains to be characterized.

RANTES/CCL5 was also up-regulated extensively in Asibi virus infected KCs when compared to mock and 17-D virus infected cells. RANTES/CCL5 is a chemotactic agent for T cells and also plays a role in activation of NK cells. Previous studies have shown that YFV infected monkeys have some lymphocyte infiltration before the final 24-hours of life in the liver (Monath et al., 1981). In contrast, YFV infected humans (at post-mortem examination) have very little if any lymphocyte infiltration in the liver (Klotz and Belt, 1930). These results, as with the IL-8, suggest the potential for significant lymphocyte infiltration into the liver; however, the lack of supporting pathology data suggests the presence of an as yet unidentified regulatory processes.

Clearly the interaction between multiple cell types, particularly KCs and hepatocytes, plays a vital role in the regulation of infection and the development of disease. In this study we focused on one component of this interaction in order to identify a specific host response that may be vital for determining whether an infection is controlled. In this study we have shown that three major pro-inflammatory cytokines are up-regulated in YFV infection of KCs, but that the wild-type Asibi virus stimulates a more profound response from these cells than does infection with the vaccine strain 17-D. This response may be correlated to the replication efficiency of Asibi virus in KCs as the infectious titer was markedly higher than that of 17-D virus. In addition, we found that IL-6 stimulated KCs had significantly attenuated IL-10 gene expression and subsequent protein release after Asibi virus infection. This effect was not seen in 17-D virus infected cells. This finding suggests that Asibi virus, in the presence of IL-6, may specifically block production of IL-10 potentially leading to an un-regulated pro-inflammatory response. Such a scenario could lead to the pathology seen in the liver of people who succumb to YFV infections. Future studies of the hepatocyte response to infection with YFV will provide additional information regarding the interactions between these two key components of the liver.

Materials and methods

Cells

Kupffer cells (KCs) were purchased from Celsis (Baltimore, MD). Due to very limited availability and highly specific donor requirements, KCs used in this experiment were from a single donor. The donor was an apparently healthy 17 year old white male with no obvious signs of liver disease and was negative for hepatitis C virus, hepatitis B virus, human immunodeficiency virus, Epstein–Barr virus and positive for cytomegalovirus. Kupffer cells arrived at a plated density of 10^6 cells/well in 24-well culture plates. This cell density was chosen by Celsis due to the small size of KCs and their standard protocol of plating cells to >70% confluency. KCs were maintained in the recommended media available from Celsis, *InVitro*GRO CP (plating) medium custom made with heat inactivated newborn calf serum and supplemented upon arrival with Torpedo antibiotic mix (Celsis). KCs media was changed upon arrival and were allowed to acclimate for 24-hours before stimulation treatments (described below). Prior to manipulation, cell density was determined (using trypan blue exclusion) in one representative well.

VERO-E6 cells were used for generation of virus stocks and titration assays. VERO-E6 cells were maintained in complete growth media (modified Eagle's medium containing 2 mM L-glutamine and Earle's balanced salts (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1% of 100× non-essential amino acids, and 1% of 100 mM sodium pyruvate (Sigma)).

IL-6 pre-stimulation of Kupffer cells

Half of the KCs described here were treated with 5 ng/mL of recombinant human IL-6 (R&D Systems) for 5 days prior to infection. The remaining half of KCs were unstimulated. IL-6 pre-stimulated and unstimulated experiments were run concurrently.

Flow cytometry

Kupffer cells (KCs) were stained for CD14, CD86 and HLA-DR and tested on a BD Biosciences FACSCanto II to determine population purity, quality, and activation state. Tests on populations were performed before infection. Antibodies used were PerCP-Cy 5.5 mouse anti-human CD14 (BD Pharmingen), PerCP-Cy 5.5 mouse IgG2a K isotype control, allophycocyanin anti-human HLA-DR, allophycocyanin mouse IgG2b isotype control (eBioscience), allophycocyanin mouse anti-human CD86 and allophycocyanin mouse IgG1 isotype control (Invitrogen).

Virus

YFV-Asibi strain was used as the wild-type virus and was provided by Dr. Robert Tesh (University of Texas Medical Branch, Galveston TX and World Reference Collection for Emerging Viruses and Arboviruses [WRCEVA]). The vaccine strain, YFV 17-D-204, was derived from the 17-D infectious clone (Rice et al., 1989) and was provided by Dr. Alan Barrett (University of Texas Medical Branch, Galveston TX). Asibi and 17-D virus working stocks were grown in VERO-E6 cells (ATCC, CRL1586) and harvested 4 days post-infection or when cytopathic effects were first visible. Viral titers were measured by immuno-focus assays.

Immuno-focus assays

Immuno-focus assays were used to determine titers of viral working stocks and in growth kinetics assays where each time point was evaluated in triplicate. Briefly VERO-E6 cells were infected with serial dilutions (10^{-1} to 10^{-6} in 100 μL) of individual samples for 1 h at 37 °C/5% CO_2 with occasional rocking. Cells were washed 3× with PBS before addition of 1 mL of 1:1 ratio of 2% agarose (ISC BioExpress): 2× MEM (Invitrogen) supplemented with 4% bovine growth serum (BGS) (Hyclone), 2% of 100× non-essential amino acids, and 2% of 100 mM sodium pyruvate (Sigma) as the overlay. Plates were allowed to incubate at 37 °C/ 5% CO_2 for 4 days before being fixed with 10% formalin for 1 h at room temperature. After fixation, the agarose plugs were removed and the plates were incubated with 70% ethanol for 20 min at room temperature. The ethanol was removed and the plates were washed once with PBS before addition of primary antibody. Mouse hyper-immune ascitic fluid raised against YFV-Asibi (WRCEVA) was used as the primary antibody. The primary antibody was diluted 1:500 in PBS containing

Fig. 3. Cytokine and gene expression from YFV infected IL-6 pre-stimulated Kupffer cells. Cytokine (left side) and cytokine gene expression (right side) were measured from IL-6 pre-stimulated Kupffer cells that were either mock (gray bars), Asibi virus (white bars) or 17-D virus (black bars) infected. Pro-inflammatory cytokines IL-8 (A), TNF-α (B) and RANTES/CCL5 (C) all had similar profiles of release and concentrations were elevated compared to the unstimulated group (Fig. 2). Asibi virus infected KCs had significantly higher concentrations of pro-inflammatory cytokines and gene expression when compared to 17-D virus infected cells. Anti-inflammatory cytokine IL-10 (D) release and gene expression was detected in higher concentrations from 17-D virus infected samples but was overall decreased when compared to the unstimulated group. Significant differences, $p<0.05$, between Asibi virus and 17-D virus infected samples are noted by ‡ for cytokine expression and * for gene expression which are centered over the time post-infection in which significance occurred. All data presented here is the average of triplicate measurements. Gene expression data is presented as fold change versus control.

5% non-fat dried milk and 1% Tween-20 (Sigma). Primary antibody was allowed to bind overnight at room temperature with constant rocking. Primary antibody was removed, plates washed once with PBS then goat anti-mouse IgG-HRP (Dako) secondary antibody diluted 1:500 in PBS containing 1% BGS was added. The secondary antibody was allowed to incubate at room temperature for 4–5 h with constant rocking. Plates were then washed thoroughly and immuno-focus units were visualized by addition of AEC substrate chromogen (Dako) for 15–20 min at room temperature. The reaction was stopped by washing with water.

Viral growth kinetics

KCs were infected with either Asibi virus or 17-D virus at a multiplicity of infection (MOI) of 0.1 diluted in complete medium. Control KCs were mock-infected with complete media. Briefly, KCs were infected with 100 μL of viral suspension for 1 h at 37 °C/5% CO_2 with occasional rocking. The KCs were then washed three times with PBS and fresh media added (1 mL). Cell culture supernatants and cell lysates were collected at 1–144 h post-infection at defined time points. Supernatants were pooled from two sample wells for each virus and treatment group per time and aliquoted into multiple tubes for growth kinetics and cytokine analysis. Two new wells were harvested at each time point due to the cells being collected for cell counts and gene expression assays (as described below). Supernatants collected for viral growth kinetics were tested in triplicate. Samples were stored at −80 °C until analyzed.

Cytokine determination

IL-8, IL-10, RANTES/CCL5 and TNF-α were detected and measured by multi-plexed magnetic bead-based format (Bio-Rad). Supernatants were not diluted before use, each tested in triplicate, and all assays were run at high sensitivity (setting on the machine to detect low concentrations of cytokines in samples) on a Bio-Plex 200 machine (Bio-Rad). The assay was performed following the manufacturer's instructions. The cytokine analysis accounted for cell death over time (represented as observed concentration per live cell in figures). Cell viability counts were collected at every time point per virus and treatment group using trypan blue exclusion.

Gene expression determination

mRNA transcripts for the above cytokines were detected and measured by a multi-plexed bead-based assay (Panomics, Inc. CA, now Affymetrix). Probes for targets were designed by Panomics, Inc. based upon sequence data available from GeneBank. Cell lysates were collected using the Panomics, Inc. lysis buffer (with proteinase K) included in the QuantiGene 2.0 Plex kits. Due to limited cell availability, only one well was used to collect lysate from each virus and treatment group per time point. All lysates were tested in triplicate and run at high sensitivity on a Bio-Plex 200 machine (Bio-Rad). Gene expression data was normalized using housekeeping genes β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The data is presented as fold change from control (mock infected) samples.

Statistical analyses

All growth kinetic, cytokine and gene expression assays reported here were performed in triplicate. A series of Student's paired *t*-tests were used to determine significant differences in viral replication, cytokine production and gene expression. Data was considered significant if $p<0.05$ (95% confidence interval).

Acknowledgments

The authors would like to thank Drs. Janice Endsley and Bersabeh Tigabu (both located at the University of Texas Medical Branch in Galveston, TX) for their helpful advice and technical assistance with the flow cytometry studies and Drs. Naomi Forrester and Melissa Whiteman (both located at the University of Texas Medical Branch in Galveston, TX) for editorial comments.

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