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

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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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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

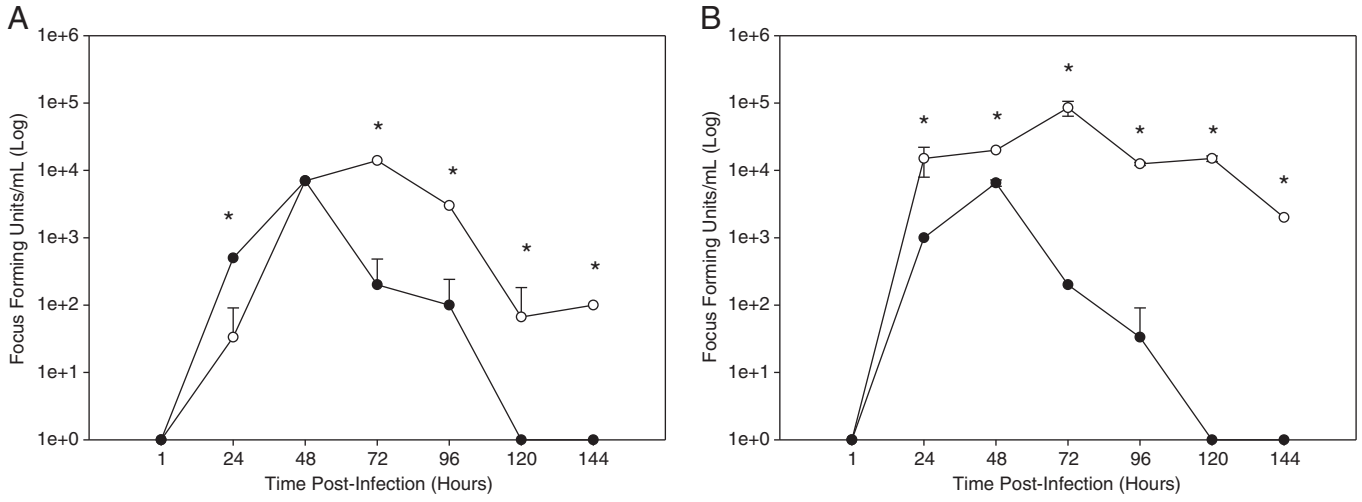


**Fig. 1.** Wild-type and vaccine strain YFV replication kinetics in unstimulated and IL-6 pre-stimulated Kupffer cells. Asibi virus and 17-D virus replication was evaluated in unstimulated (A) and IL-6 pre-stimulated (B) Kupffer cells by immuno-focus assay. Asibi virus (○) replicates to significantly (* 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.