Chapter 5
DISCUSSION

It is believed that both viral and host factors contribute to the wide spectrum of clinical manifestations of hepatitis E virus infection. Both human and animal studies have suggested that it is the immune response rather than the viral damage to hepatocytes that reflects in the outcome of hepatitis E including self limiting acute viral hepatitis and acute liver failure. The coordination of innate immune signaling and efficient expression of antiviral effector genes are necessary in governing the outcome of virus infection and immunity. Intrahepatic global gene response to HEV infection has been studied in chimpanzee model by taking serial liver biopsies (Yu et al., 2010). There are few studies documenting immunopathogenesis in HEV infection focusing mainly on adaptive immune response to the infection (Wedemeyer et al., 2013; Krain et al., 2014). However, innate immune response during HEV infection still remains underexplored area. Innate immune system is the major contributor of acute inflammation induced by microbial infections (Takeuchi and Akira, 2010). Though macrophages and DCs play important role in development of this response, nonprofessional cells such as, epithelial cells, endothelial cells, and fibroblasts also contribute significantly (Yokota et al., 2010). It is not yet known how HEV overcomes effects of host cell antiviral responses in the initial phases of establishment in host cells. Lack of efficient cell culture model system for HEV is the major hurdle in undertaking such studies.

Our search for appropriate cell culture system for analyzing innate immune response against HEV started with screening of human liver cell lines. The easiest option was to use established hepatoma cell lines (Huh7, HepG2, PLC/PRF5) or the subclonal hepatoma cells (S10-3, HepG2/C3A and Huh7.5), which were previously used for HEV replication studies (Emerson et al., 2004, 2010). We also included non-hepatic cell human lines, shown to support HEV replication (Caco2: human colon carcinoma and A549: human lung carcinoma) in our initial screening. It was realized that, though hepatoma cells were supporting HEV replication, their responsiveness to virus infection was impaired (probable reason for making them permissive to HEV), hence none could be used. Of the other two cell lines, A549 was found to be suitable for the study as it was permissive for HEV as well as responsive to dsRNA exposure (replicative intermediate of RNA viruses). We assumed that as pattern recognition receptors are highly conserved

in different tissues, it should not make any difference whether we use hepatocytes or lung cells to analyze initial antiviral cellular response. Although, it was always kept in mind that there will be tissue/cell specific factors which may influence this response. There are recent reports proving that innate immune response functions in a tissue-specific manner to control virus replication and restrict tissue tropism (Suthar et al., 2013). We carried our experiments to analyze response in HEV infected A549 cells and in parallel analysed factors responsible for the non-responsiveness of hepatoma cell lines to HEV infection.

Analysis of the response of A549 cells upon HEV infection

Significant up-regulations of inflammatory chemokine genes, RANTES and CCL20 were seen in HEV infected A549 cells (Table 4). CCL20 has been shown to mediate recruitment of CCR6 (CCL20 receptor) expressing leukocytes early upon infections. CCR6 is present on surface of immature DCs, B and subsets of T cells including effector/memory T cells, Th17 and T regulatory cells. Several studies have identified major contribution of CCL20/CCR6 interactions in the pathology of inflammatory conditions and also in amplifying local immune response in inflamed liver (Shimizu et al., 2001). RANTES is known to be secreted by fibroblasts and epithelial cells in viral infections resulting in enhanced leukocyte recruitment. Intra-hepatic expression of RANTES has been positively correlated with the severity of hepatic inflammation in chronic hepatitis C (Soo et al., 2002). CCL20 and RANTES require more attention to see whether they have any role in HEV induced liver inflammation.

Secretion of IL-6, IL-8 and TNF-α, in cells infected with HEV or cells infected UV inactivated HEV suggested involvement of HEV capsid. Inability of the soluble capsid protein to elicit antiviral response, however, confirmed that cell receptors recognized viral capsid structure and not the soluble protein (pORF2) as ‘danger signal’. On the other hand, significantly higher levels of above mentioned inflammatory genes in live HEV infected cells as compared to HEV-UV indicated involvement of additional viral molecules, synthesized during replication, in enhancing the initial signal triggered by binding of the capsids. Viruses such as influenza, HIV-1, HTLV-1 and HBV are known to trigger cellular response leading to the first wave of cytokine production even with a brief exposure of cells (Mogensen and Paludan, 2001). HEV does not induce apoptosis in infected cells and it is proposed that liver damage in HEV infection is immune mediated. Prabhu et al. (2011) have shown adequate presence of CD8+ T cells in

the liver biopsies of HEV infected patients suggesting their major role in HEV pathogenesis. Robust inflammatory response initiated at the early stages of HEV infection, as seen in our results, has potential to initiate massive infiltration of lymphocytes in the liver resulting into immune mediated damage to the tissue.

Induction of cell surface TLRs, TLR2 and TLR4, with live HEV (to higher levels) and with HEV-UV (to lower levels) directed us to speculate probable involvement of these receptors in recognizing the viral capsid. It is documented that TLR2 and TLR4 recognize viral capsid proteins and envelope glycoproteins in measles virus, HCV, murine leukemia virus, mouse mammary tumor virus and coxsackievirus B4 virus infections (Jensen and Thomsen, 2012). Parallel up-regulation of IRAK2 levels in live HEV infected cells supported our speculation of TLR involvement as IRAK2 has been shown to interact with TRAF6 and MyD88 to activate NF-κB (Kawagoe et al., 2008). Significant up-regulation of A20 transcripts in live HEV infected cells (12-96h) suggested possible autoregulation of NF-kB signaling to keep check on the inflammatory signal and limit level of response (Table 4). NF-kB induced A20/TNFAIP3 gene is known to down-regulate NF-kB signaling through cooperative activity of its two ubiquitin-editing domains (Wertz et al., 2004).

There was a low level transcriptional activation of IFN-α and IFN-ω genes, and a higher level activation of IFN-β gene with live HEV infection. Similarly, number of ISGs were up-regulated (to a lower level) only with live HEV (Table 4). These results suggested involvement of TLR3/ RLRs in detecting HEV dsRNA intermediates during replication to activate IRF3. IRF3 is constitutively expressed in all cell types, whereas, expression of IRF7 requires IFN α/β triggered pathway activation in non-lymphoid cells and DCs (Sato, et al., 1998). However, we could not detect any secreted interferons in the cell supernatants, especially IFN-β, since its transcriptional induction was higher (125, 221 and 122 folds at 48, 72 and 96 h respectively, p.i.). Higher level of transcripts and no protein synthesis for IFN-β suggested additional mechanism regulating translation of selective mRNAs in HEV infected cells. A recent study has documented HEV ORF3 mediated inhibition of STAT1 phosphorylation to down-regulate IFN-α signaling in A549 cells (Dong et al., 2012).

All TLRs except TLR3, initiate signaling through MyD88 adaptor while TLR3, endosomal dsRNA sensor, recruits TRIF which is also shared by TLR4 (Thompson et al.,

2011). RIG-I and MDA5 interact with MAVS and initiate downstream signaling pathways (Lee and Kim, 2007). Our siRNA experiments convincingly showed dependence of IL-6 and RANTES expression on MyD88 pathway in HEV infected cells. This suggested involvement of TLR2 and/or TLR4 in sensing HEV capsids (Figure 11). While, IL-8 expression was reduced by either MyD88 or TRIF knockdown (~40%, in both adaptors) (Figure 11B, 12C), indicating possible involvement of TLR3 and/or TLR4 in sensing HEV.

MAVS knockdown surprisingly resulted in increased secretion of IL-6, IL-8 and RANTES by HEV infected cells, without increasing the IFN secretion. MAVS being the sole adaptor for all RLRs, these results indicated absence of RLR trigger in eliciting antiviral response against HEV in A549 cells. On the contrary, it indicated that HEV recruits MAVS/MAVS mediated pathways in keeping check on the inflammatory response.

HEV replicates its genome via dsRNA intermediates which are expected to be sensed either by RLRs (RIG-I/MDA5) or by TLR3. Our results indicated that HEV manages to curb RLR mediated innate immune response. This could be due to, i) HEV replication occurs in endosomal compartments within the cells where dsRNA remains undetected by the cytosolic sensors or ii) HEV actively down-regulates MAVS mediated response. There is indirect evidence that HEV replicates in the endoplasmic reticulum (Rehman et al., 2008). Our observations showing involvement of both TRIF and MyD88 suggest that, dsRNA synthesized during HEV replication is detected by TLR3 present in the endosomal compartments leading to TRIF mediated IFN/ inflammatory gene activation. Complete absence of transcriptional induction of IFN genes/ISGs with HEV-UV supports this postulate. We have recently documented deubiquitination and deISGylation activity of papain like cysteine protease (PCP) domain of HEV ORF1 (Karpe and Lole, 2011). N-terminal caspase activation recruitment domain (CARD) of RIG-I is known to undergo robust ubiquitination and activation, initiating antiviral response in mammalian cells (Gack et al., 2007). It would be interesting to see whether HEV PCP has any role in down-regulating RLR mediated signaling.

These experiments with A549 HEV infections showed that HEV associated PAMPs are probably detected by TLR2, TLR3 and TLR4, which results into MyD88 and TRIF mediated downstream pathway activation resulting into activation and nuclear

translocation of IRF3 and NF-κB transcription factors to initiate transcription of inflammatory genes, IFN and interferon stimulated genes. It is known that innate response plays a major role in shaping up adaptive response in viral infections.

Analysis of antiviral responses in HEV infected hepatoma cells

Being hepatotropic virus, it was essential to use cells of liver origin for comparing our findings with A549 cells. We faced several obstacles while assessing hepatoma cell lines for HEV replication as well as finding their responsiveness to the virus. Since, HEV does not cause any cytopathic effects in infected cells and replicates with very low efficiency, it was not possible to measure its replication efficiency using any of the available tools such as plaque assay or real time PCR assay. Availability of HEV subgenomic replicon prompted us to use it for testing replication efficiency in the hepatoma cells (HepG2/C3A, Huh7.5 and S10 3). This HEV subgenomic replicon has capsid protein encoding gene replaced with luciferase reporter gene (Cao et al., 2010). Replicon can successfully complete negative strand synthesis followed by synthesis of positive sense RNA (both genomic and subgenomic). On translation of the subgenomic RNA, it yields Renilla luciferase enzyme. Measurement of luciferase activity thus directly reflects level of the virus replication; however, due to absence of ORF2/ ORF3 proteins no virus particles are formed. It is expected that this subgenomic replicon replicates in the endosomal compartment, since it is known that HEV RdRp has ability to locate itself in ER (probable site of HEV replication) (Rehman et al., 2008).

We could see efficient HEV replication in Huh-7 derived cell lines S10-3 and Huh7.5 and a significantly low level replication in HepG2/C3A. This was in agreement with the previous report (Emerson et al., 2004). Considering innate immune response as the first level of defense activated by host cells in response to virus infection (Goodbourn and Randall, 2008; Jensen and Thomsen, 2012), we questioned whether antiviral responses generated in hepatoma cell lines were responsible for variable replication efficiencies in different cell lines.

As expected, HepG2/C3A cells showed higher level transcriptional activation of pattern recognition receptor genes (PRRs) and IFN stimulated genes (ISGs) as compared to S10-3 and Huh7.5 cells, upon transfection with capped transcripts generated from the HEV Rluc replicon (Table 5). Double stranded RNA formed during replication of both

negative and positive sense RNA viruses, is known to be the major viral PAMP recognized by cells. Treatment of mammalian cells with synthetic dsRNA analog, poly(I:C), induces type I IFN synthesis through activation of transcription factors IRF-3 and NF-κB (Levy and Marie, 2004). Knowing that, in vitro generated capped transcripts may have few molecules with 5'PPP ends, which could act as danger signals to excite RLRs, we generated HEV Rluc GAA mutant replicon and used transcripts generated from this replicon as negative control in our RNA transfection experiments. The levels of above mentioned genes in HepG2/C3A cells transfected with HEV Rluc RNA were significantly higher than those with HEV Rluc GAA replicon RNA. This indicated that these cells were triggered by the viral replication intermediates (dsRNA) to induce downstream antiviral pathway genes (Table 5). None of the three hepatoma cell lines showed higher level induction of IFN-β gene, or detectable levels of inflammatory chemokines/ cytokines (IL-6, IL-8, TNF-α and RANTES) as observed in A549 cells after HEV infection. A549 cells showed more robust response in terms of magnitude as well as number of activated genes as compared to HepG2/C3A cells. A point noteworthy here is, HepG2/C3A cells, when stimulated with poly (I:C) did not secrete any detectable levels of IFNs, on contrary, A549 cells were highly responsive to poly (I:C) (Figure 6). It can be concluded on the basis of these observations that antiviral response functions in a tissue-specific manner and probably also contributes in restricting tissue tropism. Viruses are known to have their own arsenal to combat antiviral response in host cells. It is likely that certain tissues like lung are more exposed to pathogens as compared to other tissues such as liver. Hence, they have evolutionarily acquired more robust mechanisms to deal with external insults. Viruses/ pathogens capable of overcoming these hurdles can successfully establish infection in such tissues (e.g. influenza virus); otherwise, pathogens lacking such counteracting mechanisms cannot establish infections in these tissues. Liver cells probably have less robust response as compared to lung cells. Point noteworthy here is microarray based analysis of immune pathway genes of serial liver biopsies from HCV and HEV infected chimpanzees revealed broader and robust response against HCV as compared to HEV suggesting important role of the pathogen in eliciting the response (Yu et al., 2010). However, major limitation of our study is that we have used established cell lines, which may have altered proteome and one has to be careful while extrapolating any findings with such cell systems.

Our analysis of hepatoma cells showed that antiviral state of HepG2/C3A cells restricted HEV replication, while, attenuated antiviral response allowed comparatively higher HEV replication in Huh7.5 and S10-3 cells. This was further confirmed by treating Huh7.5 cells with IFN-α to see whether IFN induced response restricts HEV. Cells could successfully generate antiviral state (by inducing IFN pathway genes) and restricted HEV replication (Figure 16). Huh7 cells are known to be unique in their ability to support replication of hepatitis A and hepatitis C virus replicons (Yi and Lemon, 2002; Lohmann et al., 1999). Huh7.5, a Huh7 subclonal cell line is highly permissive for HCV replication (Blight et al., 2002) as compared to the parental Huh7 cell line. A mutation in the first CARD-homology domain of RIG-I inhibits PAMP signaling required for IRF3 activation in these cells (Sumpter et al., 2005). Our observations confirmed that Huh7.5 cells, with impaired RIG-I function, are not able to mount antiviral response upon HEV infection. However, when stimulated with IFN, in paracrine manner, the cells develop antiviral status (via Jak-Stat pathway) at par with the HepG2/C3A cells.

RIG-I resides in cytoplasm and binds to viral dsRNA probably via its RNA helicase domain. This results in activation of IRF-3 and NF-κB transcription factors via interactions of CARD-homology domains (Yoneyama et al., 2004). Pattern recognition receptor (PRR) molecules recognizing HEV associated molecular patterns are not known and considering defective RIG-I signaling in Huh7.5 cells, we thought it would be most suitable model to evaluate role RIG-I in sensing HEV dsRNA intermediates. We supplemented Huh7.5 cells with functional RIG-I and analyzed HEV replication. Cells mounted protective response (in terms of antiviral effector ISGs) and restricted HEV, confirming involvement of RIG-I in sensing HEV dsRNA (Figure 17). These results demonstrate that the RLR and interferon stimulated gene signaling cascade plays an essential role in protection against HEV infection in hepatocytes. We also introduced functional TLR3 gene in Huh7.5 cells to evaluate its role, and observed no effect of over expression of TLR3 on HEV replication (Figure 17). This confirmed that HEV replication intermediates are primarily detected by RIG-I and not by TLR3.

TLR3, RIG-I and MDA5 are known to activate TBK1/IKKε mediated IRF-3 dependent signaling pathway. Under normal conditions IRF-3 remains in cytoplasm, which, after TBK1/ IKKε mediated phosphorylation, dimerizes (either with itself or with IRF7) and translocates into nucleus to induce transcription of IFNs and ISGs (Hiscott,

2007). In order to prove our hypothesis, that innate immune signaling inhibits HEV RNA replication, we utilized a pharmacological compound BX795, which is a known inhibitor of TBK1/IKKε complex and was shown to enhance lentiviral transduction efficiency in human and mouse cell lines (Sutlu et al., 2012, Clark et al., 2009). We treated HepG2/C3A cells with BX795 to see whether it can inhibit/ reduce expression of antiviral effector genes. BX795 suppressed activation of IRF-3 and transcription of ISGs resulting into improved HEV RNA replication (Figure 18-19). In addition, rescue of BX795 effect on HEV replication with exogenously added IFN-α further confirmed importance of IRF-3 mediated innate signaling pathway in generating protective response against HEV in these hepatoma cells.

There was no detectable type I IFN secretion by HepG2/3A upon HEV infection. However, treatment of the HepG2/C3A cells with anti-IFN-β antibodies marginally increased HEV RNA replication suggesting that undetectable levels of secreted type I IFNs were also contributing in generating overall antiviral state in HepG2/C3A cells (Figure 20). Up-regulation of IRF7 and IRF9 in HEV RNA transfected cells (Table 5) also suggested this possibility. Though several human hepatoma cell lines such as HepG2, PLC/PRF5 and Huh7 and non-hepatoma cell lines Caco2 (human colon carcinoma) and A549 (lung cancer cells) have been reported to support replication of genotype 1 HEV (Emerson et al., 2004, 2010), none of these cell culture systems can provide high titers of infectious HEV in culture supernatant. Our observations with use of BX795 and anti-IFN-β antibodies in HepG2/C3A, showing improved HEV replication are promising. It would be worthwhile to test this model system for improving virus release. This improved model system can be used to understand HEV replication, ORF1 processing and to study host-virus interactions.

In conclusion, we observed that innate immune response functions in a tissue specific manner. Human lung carcinoma cells (A549) were found to be more responsive to HEV replication and mounted robust inflammatory response to restrict the virus. While, hepatoma cells responded in a relatively less robust manner. Hepatoma cells used RIG-I mediated pathways to induce antiviral effector genes and restricted the virus. However, hepatoma cells were not identical in their response, probably due to impaired innate immune pathways. Inhibition of IRF3 mediated innate immune signaling by pharmacological inhibitor BX795 and additional block by anti-IFN-β antibodies

significantly improved HEV replication. HepG2/C3A cell system, commonly used in evaluating HEV infectivity can be improved using these inhibitors and used as a model system in future HEV studies.