Discussion
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Hepatitis E, a major public health concern in developing countries, has two characteristic features:

(1) High mortality among pregnant women increasing with the duration of pregnancy and

(2) Predilection for young adults

So far, no concrete evidence has been provided to explain these features observed for several decades (for India, at least since 1955 till date). This study deals with the first feature of hepatitis E. Before proceeding further, it is important to note that in the sporadic setting, both men and non-pregnant women develop fulminant hepatitis E, a proportion succumbing to the infection (Chadha et al, 2003).

In the absence of specific antivirals, most viral infections are treated symptomatically and for the known self-limiting viral diseases; do not cause anxiety in the minds of patients and the relatives as well as for the health authorities. However, when certain high-risk groups progressing to serious consequences and are mortality are identified, it becomes obligatory to understand the factors differentiating a self-limiting infection and serious disease outcome. As far as hepatitis E is concerned, the progress in this direction has been really limited, primarily because of the absence of a robust cell culture system or small laboratory animal models. HEV was shown to infect chimpanzees (Yu et al, 2010; Krawczynski et al, 2011) as well as other primate species (Arankalle et al, 1993a, 1993b; 1995, 2007, 2009, Tsarev et al, 1995; Huang et al, 2004 & 2011).

As our institute has a facility for the experimentation in monkeys, as early as 1989, transmission experiments were undertaken mainly in rhesus monkeys. Following successful transmission of HEV to rhesus monkeys, the first obvious question was as to whether (1) the pregnant rhesus monkeys develop severe liver disease and (2) mortality is observed in these monkeys. The infection of pregnant rhesus monkeys with HEV in the third Para did not lead to severe disease or mortality eliminating the use of this model in the understanding the
disease pathogenesis (Arankalle et al, 1993). Similar results were independently reported by Dr Purcell's group (1995). Thus, one needed to depend on the studies in humans.

For studies in humans, we need viral antigens / purified virus as well as several other standardized techniques to generate meaningful data. In the absence of cell-culture-based virus, only after expression of the recombinant capsid ORF2 protein, such studies could be initiated. The other limitation has been the viral hepatitis reporting system in the endemic countries where such studies are possible. Epidemics are usually not reported or reported late. Certain techniques require immediate processing of the samples that becomes difficult or almost impossible as even minimum required laboratory facilities are usually not available at such places. With these limitations in mind, our laboratory initiated work on the pathogenesis of HEV infection (Saravanabalaji et al, 2009; Das et al, 2012; Mishra et al, 2011 and 2013; Tripathy et al, 2012a and 2012b). Though we were keen to include the patients of fulminant hepatitis E, during the course of this study, it was not possible to collect fresh samples from these patients and hence not studied. Therefore, this study examines the effect of pregnancy in self-limiting hepatitis E that includes subclinical infections as well.

Pregnancy is known to induce immune alterations to protect the foetus from rejection and the mother from infections. As innate immunity is the first line of defence and plays crucial role during pregnancy, we decided to first explore the role of TLRs in HEV infection and extend further to understand the effect of pregnancy. For this, involvement of TLRs was examined in clinical and subclinical infections among non-pregnant and pregnant individuals. As TLR levels were independent of sex, the non-ANC category included males and females. The non-ANC patients investigated during different phases of the disease showed temporal activation of TLR4 and TLR7/8 providing definite evidence for the role of these TLRs in recovery (figure 4.1-4.3). A clear difference emerged when the patients were compared with respect to the pregnancy status (figure 4.1-4.3). The ANC patients exhibited significantly lower levels of all the TLRs examined suggestive of impaired TLR response. Thus, the non-ANC and
ANC patients were distinctly different with respect to the TLR response during HEV infection.

As we had included controls representing comparable trimesters of pregnancy, we could compare the levels among pregnant women with respect to pregnancy leading to some crucial information. We showed diminished levels of TLRs in ANC controls meaning thereby that this was the impact of pregnancy. Clearly, as against the non-ANC counterparts, HEV infection in such pregnant women could not induce the expression of TLRs, the crucial modulators of innate immunity. TLR4 is known to be associated with recovery and protection from infection with respiratory syncitial virus (Cyr et al, 2009) whereas TLR7 is known to recognize RNA viruses (Jennifer et al, 2004; Arankalle et al, 2010). The innate immune response to the fusion protein of an important respiratory pathogen of humans, respiratory syncytial virus (RSV), was mediated by TLR4 and CD14. In experiments with TLR4-deficient mice, Kurt-Jones et al (2000) showed that RSV persisted longer in the lungs of infected TLR4-/-mice compared to the wild type. Thus TLR4 and CD14 were associated with recovery and protection from infection with RSV.

To address the role of TLR4 in the innate immune response to a respiratory virus infection, Haynes et al examined activity of NK cells and CD14+ cells in the TLR4-deficient (TLR4-/-) C57BL/10ScNcr and wild-type C57BL/10Sn mice (TLR4+/-) infected with RSV or influenza virus. The TLR4 deficient mice showed diminished function of NK cells and CD14+ cells and also impaired IL12 production, since influenza virus-infected TLR4null mice did not display altered immune cell trafficking or impaired NK cytotoxicity compared to the TLR4wt mice, similar to the observations following RSV infection of these mice (Haynes et al, 2001).

Arcangeletti et al showed that HCMV infection of THP-1 macrophages leads to TLR 4 activation and suggested TLR4 as a mediator of HCMV-triggered cell cycle activation in THP-1 macrophages, in turn favouring the development of an efficient viral lytic cycle (Arcangeletti et al, 2013). Arankalle et al showed the activation of TLR4 in the PBMCs of pandemic H1N1 2009 virus infected patients (Arankalle et al, 2009).
TLR7 is known to recognize RNA viruses like Influenza A virus (Diebold et al, 2004; Jennifer et al, 2004; Arankalle et al, 2009; Liu et al, 2012). Jeisy-Scott et al (2011) showed that myeloid derived suppressor cells (MDSC) accumulate in the lungs of TLR7 deficient mice and expressing higher levels of cytokines as compare to wild-type mice. Furthermore, the CD4$^+$ T cells of TLR7(−/−) mice expressed higher levels of Th2 type of cytokines as compared to wild type and leading to a Th2 humoral response, suggesting that TLR7 modulates the accumulation of MDSCs during an IAV infection in mice, and that lack of TLR7 signalling leads to a Th2-biased response (Jeisy-Scott et al, 2011).

Kaminski et al strongly suggested that TLR7-mediated sensing of influenza viruses by pDCs substantially contributes to antiviral protection, presumably by enhancing the synthesis of type I and type III IFN in the infected host. The viral lung titres of MyD88-deficient, TLR7-deficient and wild-type mice were similar at day 2 post-infection, whereas titre differences increased during the following days during which MyD88- or TLR7-deficient mice cleared the virus less efficiently than the wild-type controls (Kaminski et al, 2012).

We did not observe an increase in the levels of TLR3 in non-ANC patients. However, type-I IFN and other pro-inflammatory cytokine responses following stimulation with polyI:C suggests TLR3 activation during HEV infection (figure 4.20-4.27). These observations indicate that the response was probably not mediated by a change in TLR expression. Importantly, these patients exhibited higher expression of TLR3 at gene level (figure 4.33). As TLR3 recognizes double-stranded RNA and HEV is a single-stranded RNA virus, it implies that in addition to hepatocytes, viral replication takes place in PBMCs as well. Elevated levels of RIG1 and MDA5 genes in non-ANC patients support this view (figure 4.32) (Kawai et al, 2007; Leung et al, 2012). Ippagunta et al, (2011) showed the presence of HEV RNA in the PBMCs of acute hepatitis E patients. Of the 44 patients with acute hepatitis E, including 27 with detectable IgM anti-HEV and 19 with detectable serum HEV RNA, 11 had detectable HEV RNA in their PBMCs. PBMC specimens with strong HEV RNA had detectable negative-strand HEV RNA, a marker of viral replication (Ippagunta et al, 2011). Further work is needed to examine if PBMCs support limited replication of HEV. In both patient
categories, an excellent correlation of the surface expression of TLR4, TLR7 and TLR8 and gene expression (figure 4.33-4.34) was observed which indicative of HEV infection affecting transcription, translation and membrane localization of these TLRs.

TLR-specific induction of type I interferons (IFNβ) characteristic of innate immune response further strengthened the protective role of TLR3 and TLR4 in Non-ANC patients (figure 4.20-4.27). HEV infection in pregnant women induced higher levels of IFNα suggestive of subtype shift in ANC category. However, the normal pregnant women induced high levels of type-I interferons (both at protein and gene levels) indicative of heightened innate response during pregnancy. Thus the increased levels among ANC patients reflect the effect of pregnancy, not altered by the viral infection. Thus, in contrast to non-ANC category, HEV infection in pregnant women did not result in further rise in the interferon levels. As these women had uneventful recovery, additional interferon secretion may not probably was necessary. The significance of the subtype shift to IFNα is not clear.

TLR-specific induction of type I interferons (IFNβ) characteristic of innate immune response further strengthened the protective role of TLR3 and TLR4 in Non-ANC patients. The data confirms impaired TLR response in HEV infection during pregnancy. Gene expression analysis identified high expression of IFNα (28.4 and 21.7 fold respectively) and IFNβ (13.3 and 13.5 fold) genes in acute non-ANC and ANC patients respectively. Though pregnancy was associated with higher expression of both the genes, HEV infection induced a further significant increase. Clearly, despite the high expression of these genes in pregnancy, the protein expression was hampered. The reasons for the specific inhibition of translation of IFNα in non-ANC patients and that of IFNβ in ANC patients are not understood. In non-ANC patients, the IFN genes continued to be expressed at high levels through convalescence while the receptor levels returned to normal thereby limiting interferon response. In contrast, the receptor expression in the acute ANC patients remained normal and may have affected interferon response. These patients were not bled during the recovery phase.
An attenuated innate antiviral immune response (significant reduction in IFN-α and IFN-λ production) was reported when PBMCs from pregnant women were stimulated in-vitro with H1N1/09. No difference of expression of TLR3, TLR7 and TLR9 were found in non pregnant and pregnant women (Forbes et al, 2012). In contrast, PBMCs from HEV-infected pregnant women induced higher levels of IFNα with all the 3 TLR-ligands and lower levels of IFNβ (TLR-3 and 4 ligands), when compared to induction of IFNβ in non-ANC patients. Thus, in the absence of significant TLR response, PBMCs from pregnant women induced higher IFNα expression than the non-ANC patients and, diminished levels of other subtypes. These results emphasize the viral-specific modulation of innate immune response in pregnant women.

TLR-induced pro-inflammatory cytokines play important role in activating different cell types. We determined the levels of 6 cytokines in the TLR-ligands stimulated PBMCs. Impaired cytokine response was observed among the acute non-ANC patients, IL1β being the only cytokine induced by all the 3 ligands (figure 4.20-4.27). IL1β is a most prominent pro-inflammatory cytokine involved in tissue inflammation and important for host immune response and viral clearance. Zhu et al showed that the replication of HCV in the replicon FCA1 cell line inhibited with IL1β stimulus invitro and the activity of IL1β was associated with ERK-MAP-kinase and IFN responsive genes activation (Zhu et al, 2003). When we consider pregnancy and hepatitis E, different patterns emerged: modulation by pregnancy, HEV not causing any change {IL1β and IL12p70 (polyI:C), IL1α, IL1β, IL6 and TNFα (R848)}, modulated by both pregnancy and HEV {IL1α, IL6, IL12p40 and TNFα (polyI:C), IL6, IL12p70 and TNFα (LPS), IL12p70 (R848)} and no effect of pregnancy {IL1α, IL1β and IL12p40 (LPS)}. Overall, impaired cytokine response was characteristic of pregnancy with hepatitis E.

To further establish the role of TLRs in HEV infection, activation of downstream signalling pathways was assessed only in the non-ANC patients (figure4.5-4.14). We determined the involvement of MyD88-dependent and independent (TRIF-mediated) pathways in hepatitis E by measuring levels of IRAK4, IKBα, NFkB and TBK1, IRF7 respectively in the TLR-ligand stimulated PBMCs. The results confirm involvement of MyD88-independent (TLR3),
MyD88-dependent and independent (TLR4) and MyD88-dependent (TLR7 and TLR8) pathways in HEV infection. We further quantitated expression of the genes associated with these pathways. Significantly higher expression (3.1-11.6 fold) of the 14/15 genes associated MyD88-dependent pathway, returning to normal during recovery, proves the role of MyD88 pathway in the protection against HEV (figure 4.35-4.35). Though we did not assess the signalling molecules in ANC patients, no induction of any of the 15 genes in these patients and controls suggests distinct possibility of lowered expression of these molecules. We chose 6 genes to evaluate the MyD88 independent pathway and found 4.2-9.9 fold higher expressions of 5 of these genes in the acute phase, returning to normal during convalescence, reconfirming involvement of this pathway in HEV infection. No change in the expression of these genes was observed in ANC controls and patients. Together with lowered TLR expression and genes in the PBMCs, these data provide definite evidence for inability of pregnant women infected with HEV to mount TLR-induced immune responses. The heat map clearly showed distinct patterns and clustering of ANC and non-ANC categories.

As the identification of subclinical infection takes time, these patients could not be studied for the flow cytometric analysis or PBMC culture-based assays requiring immediate processing. Based on the gene expression analysis, the subclinical infections in non-ANC and ANC patients were almost similar to the clinical disease.

Another important finding of our investigations is documentation of the effect of pregnancy on the TLR-associated immune pathways among rural women from Maharashtra, western India. Considering the fact that pregnancy requires a unique immunological balance to accommodate the foetus and to protect the mother against invading pathogens, these observations are significant. Despite normal expression of the respective genes, diminished TLR2, TLR3, TLR4 and TLR8 levels suggest translation inhibition in the PBMCs of the pregnant women. At gene level, pregnancy did not influence the expression of both MyD88 dependent and independent pathways and TLR genes, except for the down-regulation of TLR2 during the first trimester. Impaired TLR4 stimulation
and higher TLR3 and TLR7/8-dependent induction of interferons / cytokines during pregnancy confirms altered innate response. Though the expression of the interferon-associated genes did not differ with the trimesters, elevated expression of IFNA1 and its receptor (1st trimester) and both interferon A and B (later trimesters) suggests up-regulation of interferon genes throughout pregnancy.

Overall, we have identified the effect of pregnancy on the TLR-mediated immune responses in HEV infection in patients with uneventful recovery. Though this study did not include fulminant hepatitis patients, a few studies including our own, dealing with other aspects have been reported in fulminant hepatitis E patients, including pregnant women (Saravanabalaji et al, 2009; Borkakoti et al, 2013; Bose et al, 2011; Jilani et al, 2007).

We earlier studied the association of viral load, anti-HEV titers and cytokine / proliferative responses of HEV-antigen-stimulated PBMCs from acute-recovering and fulminant hepatitis patients. Except one all FHF patients showed undetected levels of viral HEV RNA and anti-HEV-IgM/ IgG titers were higher in the FHF than acute patients. As compared to AVH, higher levels of both Th1 and Th2 cytokines were observed in FHF patients (Saravanabalaji et al, 2009). This study further led to the study of polymorphism of TNFα and IFNγ cytokines during different clinical presentations of hepatitis E (Mishra & Arankalle, 2011). In this study, we focused on the cell enumeration analysis of two important immune cell types and respective subpopulations, i.e., CD14+ monocytes crucial for both innate and adaptive immune response (Gordon & Taylor, 2005) and CD4+ T helper cells that play an important role in the adaptive immune system, in self-recovering hepatitis E patients with or without pregnancy.

Circulating monocytes are primary targets for viruses (Ströher et al, 2001; Van de Sandt et al, 2012; Torres et al, 2013; Azeredo et al, 2010; Kurt-Jones et al, 2000). Kurt-Jones et al (2000) demonstrated that CD14+ monocytes cells recognized RSV infection and generated TLR 4 mediated immune response. The activation of monocytes was triggered by Marburg and Ebola viruses, as indicated by the release of the pro-inflammatory cytokines IL-1β, TNF α, and IL6 as well as the chemokines IL-8 and GRO-α (Ströher et al, 2001). Ströher et al
suggested that infected monocytes may play an important role in the spread of filoviruses and in the pathogenesis of filoviral hemorrhagic disease (Ströher et al, 2001).

Influenza virus infection activates innate and adaptive immune system via the activation of monocytes (Van de Sandt et al, 2012). Azeredo et al showed that the elevated plasma levels of pro-inflammatory cytokines significantly correlated with the increase in classical monocytes (CD14⁺/CD16⁺) during dengue virus infection (Azeredo et al, 2010). In another study, Torres et al (2013) showed impaired monocyte response during dengue virus infection. Patients infected with dengue virus showed altered expression of CD80 and CD86, and affected the immune response (Torres et al, 2013).

Monocytes and neutrophils are the primary phagocytes and the reduced number of these cells leads to increased susceptibility to microbial infections. The raised frequency of CD14⁺ monocytes in healthy pregnant women (p<0.001; figure 4.15-4.16) suggests heightened phagocytic barrier, a required immune alteration during healthy pregnancy. Kraus et al (2012) showed a significant increase in the blood phagocytes and pDCs during pregnancy and decrease in the frequency and activity of NK and T cells. Alterations were also observed in the antimicrobial proteins and serum cytokines. Thus, pregnancy is not a period of immunosuppression but an alteration in immune priorities characterized by a strengthening of innate immune barriers and a concomitant reduction in adaptive/inflammatory immunity in the later stages of pregnancy (Kraus et al, 2012). Though non-ANC patients exhibited higher frequency of CD14⁺ monocytes, the disease in ANC category was characterized by a further increase in the proportion of CD14⁺ monocytes (p<0.01) providing evidence for the role of monocytes in recovery from HEV infection. Importantly, the monocyte frequency in pregnancy did not significantly increase due to HEV infection.

Considering a central role of monocytes in the innate response, we examined the frequency of co-expression of TLR2 and TLR4 receptors on the CD14⁺ cells and showed for the first time, higher frequency of CD14⁺ monocytes (and higher mRNA expression) along with higher co-expression of TLR2 and TLR4 in non-ANC patients (figure 4.16). Earlier, we showed elevation of TLR4,
TLR7 and TLR8 in the PBMCs of the non-ANC patient, while a significant decline was noted in the ANC patients. It is plausible that during HEV infection, TLR2 is restricted to CD14+ monocytes while TLR4 is present on other immune cells and hence the difference in PBMC and monocyte analyses. Overall, the results suggest involvement of CD14 and TLR4/TLR2 in the activation of monocytes. Kurt-Jones et al showed recognition of RSV infection by CD14 and TLR4 and produced inflammatory innate response (Kurt-Jones et al, 2000). Azeredo et al showed increase in CD14+TLR2+ and CD14+TLR4+ cells during dengue virus infections (Azeredo et al, 2010).

The disease in ANC category was associated with a significant reduction in the frequencies of CD14+TLR2+ and CD14+TLR4+ monocytes (p<0.001 for both) as compared to the non-ANC patients. Considering the lower proportion of CD14+TLR4+ cells (p<0.001) and unaltered frequency of CD14+TLR+ monocytes (p>0.1) in the control pregnant women, it may be surmised that HEV infection in pregnancy did influence CD14+TLR2+ cells and overall suggestive of impaired innate immune response during HEV infection in pregnancy. A significantly lower TLR mRNA levels in the ANC patients than in the non-ANC patients was shown earlier by us. Taken together, these observations suggest reduced activation of monocytes via TLR4 and TLR2 during hepatitis E infection in pregnancy.

We further analysed the activation markers on the CD14+ monocytes, driving the adaptive immunity. In the non-ANC patients, significantly higher expression of co-stimulatory molecules such as CD80, CD86 and HLA-DR at protein and mRNA levels suggested classical activation of the monocytes, antigen uptake and presentation (figure 4.15-4.17 & 4.41-4.42). We have previously shown higher expression of CD11c+/CD86+ cells during acute HEV infection (Das et al, 2013). The higher levels of inflammatory cytokines in the plasma of acute hepatitis E patients further support circulation of the activated CD14+ monocytes, an indication of inflammatory response during HEV infection.

A comparison of the healthy controls showed that the phenotype in the pregnant women was characterized by increased and normal expression of the co-stimulatory molecules CD80 and CD86 respectively, and lower expression of HLA-DR. Following clinical hepatitis E, no significant difference was noted
(p>0.1). However, as compared to the non-ANC patients a significant reduction in the expression of CD86 and HLA-DR and increased frequency of CD80 indicate reduced antigen-presenting ability due to the incomplete up-regulation of the co-stimulatory receptors. Interestingly, in contrast to concomitant increase in the mRNA levels in ANC controls (except HLA-DR), in the ANC patients, higher mRNA levels of CD86 and HLA-DR did not result in higher protein expression. Inhibition of the translation of these proteins seems a distinct possibility. These observations need to be confirmed by assessing HEV-specific immune responses in pregnant women with disease.

DC-SIGN (CD 209) is a C-type lectin receptor present on both macrophages and dendritic cells. DC-SIGN on macrophages recognizes and binds to glycoproteins present on the virus capsid (Khoo et al, 2008; Geijtenbeek et al, 2000). Despite increased mRNA levels (figure 4.42), the unaltered expression of CD14+CD209+ cells in acute non-ANC patients suggests no role of CD209+ monocytes in HEV infection. Though the ANC controls showed higher proportion of CD14+CD209+ cells (p<0.001), the ANC-patients circulated normal proportion of these cells negating the role in HEV infection during pregnancy. The possibility of enhanced binding of viral capsid in pregnant women with increased CD209+ cells cannot be ruled out.

The next aim was to examine phenotypic activation of T helper cells during HEV infection. We confirmed the unaltered proportion of CD4+ T cells in self-recovering non-ANC patients (Tripathy et al, 2012) and extended the same to acute-ANC patients and the corresponding controls. A lowered CD4+ T cell count was shown in ANC patients with fatal outcome (Jilani et al, 2007). We further showed increased levels of CD4+CD137+, CD4+CD152+ and CD4+CD278+ cells in the non-ANC patients (figure 4.18-4.19). CD137 co-stimulation has been shown to impact both CD4+ and CD8+ cell functions (Myers et al, 2005). Cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) is considered to be critical for Treg suppressive function (Flores-Borja et al, 2008). Previously our group demonstrated association of Treg cells in acute HEV infection (Tripathy et al, 2012). The increased frequency of CD4+152+ cells and up-regulation at gene levels support the role of T reg in acute HEV infection. The higher phenotypic
expression of CD4+CD278+ T cells indicates T cells activation. With the involvement of CD278 in the IgG class switching (Dong et al., 2001; Rudd & Schneider, 2003; McAdam et al., 2001), it is pertinent to note that IgG subclass switching in hepatitis E was recently shown by us (Deshmukh et al., 2013).

The healthy pregnant women expressed higher levels of CD4+152+ and CD4+278+ cells while CD4+CD137+ cells were comparable with the non-pregnant controls. Importantly, following HEV infection, levels of all the three T cell subtypes were comparable in ANC and non-ANC patients suggesting similar response in both patients groups.

In accordance with the activated CD14+ monocytes or/and activated T cells, we observed higher levels of circulating inflammatory cytokines (IL12, TNFα, IL6 and IL8) in the acute hepatitis E patients. On contrary, impaired response was evident in the ANC patients as judged by the diminished levels of all the 4 cytokines when compared to the non-ANC patients. However, when ANC-controls were used for comparison, higher secretion of IL12 and TNFα by HEV infection was apparent (figure 4.28-4.31). These results point towards impaired immune response in hepatitis E during pregnancy.

Overall, the present study dealing with self-recovering hepatitis E with or without pregnancy identified the effect of pregnancy on the TLR-mediated immune responses in HEV infection. Based on cell enumeration analysis, we conclude that as against the classical activation of CD14+ monocytes in the non-ANC patients, impaired response was evident in the ANC-patients while the CD4+ T cell populations were similar in both patients groups. We would very much like to extend these observations to fulminant hepatitis patients in ANC and non-ANC categories progressing either to recovery or death.