Human Immunology 80 (2019) 855–862

Contents lists available at ScienceDirect

Human Immunology

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

Phenotypic analysis of monocytes and CD4$^+$ T cells in hepatitis E patients with or without pregnancy

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A B S T R A C T

High mortality in pregnant women is a characteristic of hepatitis E virus (HEV) infection. Role of monocytes/T cells in HEV infection during pregnancy is still unclear. We compared CD14$^+$monocytes and CD4$^+$T cells by flow-cytometry in hepatitis-E patients including 13 pregnant (Antenatal care, ANC), 25 non-ANC patients and respective controls (12 and 20). Non-ANC-patients showed significantly higher frequency of monocytes with increased expression of CD80, CD86 and HLA-DR than control individuals ($p < 0.001$).

Healthy pregnancy was associated with increased frequency of monocytes with higher CD80 expression and lower levels of HLA-DR ($p < 0.05$) compared to non-ANC controls. ANC-patients exhibited elevated levels of monocytes ($p < 0.01$) with higher expression of CD80 ($p < 0.001$) and reduced levels of HLA-DR and CD86 ($p < 0.05$) when compared with non-ANC patients. TLR2 and TLR4 surface expression on monocytes was higher in non-ANC-patients ($p < 0.00$) and lower in the ANC-patients ($p < 0.01$). Healthy-ANCs exhibited lower TLR4 expression on monocytes ($p < 0.05$). HEV infection did not change the frequency of CD4$^+$ and CD4$^+$CD28$^+$T cells in patients' group ($p > 0.05$). Compared to respective controls, CD137$^+$ and CD152$^+$CD4$^+$T cells were higher ($p < 0.05$) in both patients' categories. Higher levels of CD152$^+$CD4$^+$T cells ($p < 0.001$) was noted in healthy pregnant women. Among patients' groups, the CD4$^+$T cells and their sub-population were not different ($p > 0.05$).

We found higher and reduced levels of circulating inflammatory cytokines (IL12, TNFα, IL6 and IL8; miliplex-assay) in non-ANC and ANC-patients respectively. In conclusion, on contrary to 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 the patient groups.

1. Introduction

Hepatitis E is a self-limiting disease caused by Hepatitis E virus (HEV). HEV is primarily transmitted via the fecal oral route [1–4]. In India, HEV contributes > 50% of acute viral hepatitis cases in adult population [5,6]. Fulminant hepatic failure in pregnancy is the severe outcome of infection with high mortality rate [7].

Pathogenesis of fulminant hepatitis E is poorly understood primarily because of the lack of a convenient small laboratory animal model and a robust, high virus-yielding cell line. As pregnancy is a state of immune alterations to accommodate the fetus, the infecting pathogens may lead to divergent immune responses, depending on the type of the pathogen, health status of the individual and pregnancy duration. A few studies on fulminant hepatitis E patients including pregnant women have been reported [8–12].

Peripheral blood monocytes infiltrate to the site of infection, are exposed to viruses that triggers their activation [13–14]. Activated monocytes express increased levels of TLRs, co-stimulatory molecules and secrete several cytokines [15–18]. Upon activation, monocytes can differentiate into macrophages and dendritic cells and orchestrate adaptive immune response by activating CD4$^+$ and CD8$^+$ T cells [19].

Previously, we showed that viral load was not associated with fulminant disease. Higher anti-HEV titers and enhanced Th1 response of HEV-antigen-stimulated PBMCs recorded in the fulminant hepatitis patients [9] led to studies of T cell response in HEV infection. We and others observed HEV specific CTL response in acute patients but could

Abbreviations: ANC, Antenatal care (pregnant women); Non-ANC, Men and non-pregnant women; ALT, Alanine transaminase; AVH, Acute viral hepatitis; HEV, Hepatitis E virus; IL, Interleukin; POD, Post onset day; TLR, Toll like receptors; TNF, Tumor necrosis factor

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https://doi.org/10.1016/j.humimm.2019.06.011
Received 15 April 2019; Received in revised form 19 May 2019; Accepted 23 June 2019
Available online 05 July 2019

not find any difference in the frequency of peripheral $CD8^+$ T cells [20–21]. Higher regulatory T cells (Tregs) response in acute hepatitis E patients was also recorded [22]. On the other hand, $CD4^+$ T cells are the main mediator of adaptive immunity and have direct effect on viral infections. We therefore focused on cell enumeration analysis of two important immune cell types and respective phenotypes, i.e., $CD14^+$ monocytes and $CD4^+$ T helper cells in self-recovering hepatitis E patients. In view of the role pregnancy in hepatitis E, the study was extended to pregnant patients as well.

2. Patients and Methods

This study was approved by the institutional biosafety and human ethical committees. An informed consent was taken from all the participants. The healthy pregnant women were bled on the request of the health authorities for the identification of IgM-anti-HEV positives so that they can be monitored for the symptoms of the disease. During epidemics the presence of bile salts/bile pigments in urine and serum ALT levels were the only biochemical markers tested for all the patients. A detailed clinical examination was done for all the acute viral hepatitis E (AVH-E) cases. All AVH-E patients had typical symptoms of acute viral hepatitis, such as sudden onset of fever, nausea, vomiting, weakness, jaundice, and elevated serum ALT levels.

2.1. Study subjects

Only patients with IgM-anti-HEV positive ELISA tests were included in the study. Table 1 provides details of the study population. The patients were categorized as: non-ANC hepatitis E patients (n = 25, non-ANC patients) and pregnant women with acute in 2nd / 3rd trimester (ANC patients, n = 13). Similarly, the healthy controls were divided into two groups non-ANC subjects (n = 20) and ANC controls (pregnant women in 2nd and 3rd trimester; n = 12). All the individuals included in the study were negative for IgM-anti-HAV, HBsAg, IgM-anti-HBc and anti-HCV antibodies (ELISA, Abbott, AXSYM System). In addition, the controls were negative for both IgM and IgG anti-HEV antibodies [23]

2.2. Flow cytometry analysis

Anti-human antibodies for different CD markers i.e. FITC-TLR2 and PE-TLR4 (Imgenex, USA), PECy7-CD4, PECy7-CD14, PE-CD28, PE-CD80, FITC-CD86, PE-CD137, PE-CD152 and FITC-HLA-DR (BD Biosciences, USA) were used. 100 µl of whole blood was processed for staining with anti-human antibodies, followed by lysis of RBCs with BD FACS lysis solution (BD Biosciences, USA), washing and fixation with 4% paraformyldehyde. Stained cells were analyzed on BD FACS Aria-II. $CD4^+$ T cells and $CD14^+$ monocytes were gated and displayed through histogram plots for other surface markers. The levels were expressed as percentage of cells (mean ± SE).

2.3. Cytokine measurements

Plasma cytokines were determined using a custom premixed Milliplex Map Kit (Millipore, USA) for 4 cytokines i.e. IL6, IL8, IL12 and TNFα on Bio-Plex Protein Array System (Bio-Rad, CA). For statistical analysis, a value of 0.2 pg/ml was used for samples having undetectable concentrations.

2.4. Gene expression analysis

Frozen PBMCs were used to extract total RNA by using Ribopure Kit (Life technologies, USA) as per the manufacturer's instructions and purity was checked for quantity and quality by Nanodrop spectrophotometer (ND-1000) and bioanalyzer (Agilent, USA) respectively. 500 ng of total RNA were used for cDNA synthesis using High Capacity cDNA RT Kit (Life technologies, USA). cDNAs were mix with equal volumes of TaqMan 2XPCR master mix (Life technologies, USA) and run on the Human custom TLDA panel (Life technologies, USA) by using 7900 Real-Time PCR system (Life technologies, USA). ΔΔCt method was used to calculate RQ value (Life technologies, USA). 18s RNA was used as an endogenous control. Genes expressed at ≥2fold higher or lower levels when compared to controls were considered as differentially modulated.

2.5. Statistical analysis

Flow cytometry and cytokine data were presented as mean ± SE. The one way ANOVA and Mann-Whitney *U* test was used for group comparisons. For all analyses, a p value of < 0.05 derived from a two tailed test was considered significant. All statistical analyses were performed with 'SPSS11.0 for Windows' software (SPSS Inc.).

3. Results

3.1. Monocyte activation during hepatitis E

We observed an increased frequency of $CD14^+$ monocytes in the non-ANC patients when compared to the non-ANC controls (9.6 ± 0.8 vs 6.1 ± 0.6; $p < 0.001$; Table 2 and Fig. 1a & g). The ANC controls exhibited elevated levels of $CD14^+$ monocytes than the non-ANC controls (10.9 ± 1.0 vs 6.1 ± 0.6; $p < 0.001$). Among the patient categories, the proportion of $CD14^+$ monocytes was significantly higher in the ANC category than the non-ANC patients (9.6 ± 0.8 vs 15.0 ± 1.7; $p < 0.01$). However, no difference was observed when ANC patients and respective controls were compared (15.0 ± 1.7 vs 10.9 ± 1.0; $p > 0.05$).

Further, we estimated the levels of co-stimulatory/activation markers present on the monocytes. As compared to the non-ANC controls, levels of $CD14^+CD80^+$, $CD14^+CD86^+$ $CD14^+HLA\text{-}DR^+$ monocytes were significantly higher in the non-ANC patients (20.7 ± 3.5 vs 33.6 ± 3.0; 56.9 ± 3.4 vs 70.5 ± 2.6 and 51.5 ± 3.6 vs 65.5 ± 3.7; $p < 0.001$, Table 2 and Fig. 1b,c,d & g). A comparison of non-ANC and ANC control groups revealed higher frequency of $CD14^+CD80^+$ (20.7 ± 3.5 vs 50.1 ± 5.1; $p < 0.001$) during pregnancy. Among controls, the expression of $CD14^+HLA\text{-}DR^+$ was diminished (9.7 ± 1.6 vs 51.5 ± 3.6; $p < 0.001$) in ANC group while $CD14^+CD86^+$ monocytes levels were comparable (47.0 ± 5.8 vs 56.9 ± 3.4; $p > 0.1$). When the patient categories were compared, ANC-patients exhibited significant reduction in the levels of $CD14^+CD86^+$ and $CD14^+HLA\text{-}DR^+$ monocytes (44.1 ± 12.7 vs

Table 1
Characteristic features of study groups.

Study groups	No	Age (mean ± SE)	Male:Female	ALT (mean ± SE)	POD (mean ± SE)	Anti-HEV IgM/IgG status
Healthy (non-ANC)	20	26.4 ± 0.8	12:08	31.7 ± 2.4	NA	-/-
Healthy ANC (2nd$^+$3rd trimester)	12	21.8 ± 0.6	00:12	29.4 ± 3.1	NA	-/-
Acute (non-ANC)	25	35.6 ± 3.5	14:11	267 ± 42.9	8.9 ± 2.1	$^+/^+$
Clinical ANC (2nd$^+$3rd trimester)	13	22.3 ± 1.1	00:13	109 ± 32.7	13.5 ± 1.9	$^+/^+$

NA, not applicable

Table 2
Levels of monocytes, $CD4^+$T cells and associated molecules (Mean ± SE) in HEV patients and corresponding controls.

CD molecules	non-ANC controls	non-ANC patients	ANC controls	ANC patients	*	**	#	†
$CD14^+$	6.1 ± 0.6	9.6 ± 0.8	10.9 ± 1.0	15.0 ± 1.7	0.0004	0.0513	0.0001	0.0023
$CD14^+$ $CD80^+$	20.7 ± 3.5	33.6 ± 3.0	50.1 ± 5.1	57.7 ± 4.7	0.0095	0.2852	0.0000	0.0001
$CD14^+$ $CD86^+$	56.9 ± 3.4	70.5 ± 2.6	47.0 ± 5.8	44.1 ± 12.7	0.0036	0.8369	0.1300	0.0085
$CD14^+$ HLA-DR^+	51.5 ± 3.6	65.5 ± 3.7	9.7 ± 1.6	8.9 ± 1.4	0.0092	0.6987	0.0000	0.0000
$CD14^+$ TLR 2^+	63.3 ± 4.8	89.9 ± 1.7	53.0 ± 5.4	56.5 ± 6.0	0.0000	0.0523	0.2128	0.0005
$CD14^+$ TLR 4^+	43.1 ± 3.4	61.2 ± 3.4	13.5 ± 4.9	14.5 ± 5.2	0.0005	0.8897	0.0000	0.0000
CD 4^+	28.0 ± 1.7	29.0 ± 1.3	32.2 ± 1.6	32.4 ± 1.1	0.6461	0.9213	0.1385	0.0961
$CD4^+$ $CD28^+$	88.6 ± 1.6	91.0 ± 1.2	85.4 ± 3.8	86.2 ± 3.1	0.2748	0.8701	0.3528	0.0961
$CD4^+$ $CD137^+$	17.9 ± 5.0	53.4 ± 5.5	28.2 ± 5.5	57.0 ± 7.1	0.0000	0.0041	0.2286	0.6997
$CD4^+$ $CD152^+$	14.3 ± 3.6	55.0 ± 5.3	30.2 ± 1.7	71.1 ± 5.1	0.0000	0.0000	0.0070	0.0657

* pValue, non-ANCcontrols Vs non-ANC patients
** pValue, ANC controls Vs ANC patients
# pValue, non-ANC controls Vs ANC controls
† non-ANC patients Vs ANC patients

70.5 ± 2.6 and 8.9 ± 1.4 vs 65.5 ± 3.7; $p < 0.01$) while $CD14^+CD80^+$ monocytes increased (57.7 ± 4.7 vs 33.6 ± 3.0; $p < 0.001$). No difference was noted in these markers when ANC patients and ANC controls were compared (Table 2).

A comparison of the expression of TLR2 and TLR4 on the monocytes revealed a significant increase in both of the TLRs in the non-ANC patients than in the non-ANC controls (89.9 ± 1.7 vs 63.3 ± 4.8 and 61.2 ± 3.4 vs 43.1 ± 3.4; $p < 0.001$, Fig. 1e,f & g). The ANC controls exhibited diminished expression of $TLR4^+$ (13.5 ± 4.9 vs 43.1 ± 3.4; $p < 0.001$) and comparable levels of $TLR2^+$ (53.0 ± 5.4 vs 63.3 ± 4.8; $p > 0.1$) monocytes respectively than in the non-ANC controls. The phenotypic frequency of TLR2 and TLR4 positive monocytes was significantly lower in the ANC patients than the non-ANC patients (56.5 ± 6.0 vs 89.9 ± 1.7 and 14.5 ± 5.2 vs 61.2 ± 3.4; $p < 0.001$). ANC patients and controls were not different in any of the TLRs examined (56.5 ± 6.0 vs 53.0 ± 5.4 and 14.5 ± 5.2 vs 13.5 ± 4.9; $p > 0.1$, Fig. 1e, f & g).

3.2. Expression of $CD4^+$ T helper cells

To assess modulation of adaptive immune response in HEV infection, we determined $CD4^+$ T helper cells and associated co-stimulatory molecules in non-ANC and ANC patients. As compared to the respective healthy controls $CD4^+CD137^+$ and $CD4^+CD152^+$ T cells were significantly higher (53.4 ± 5.5 vs 17.9 ± 5.0; 55.0 ± 5.3 vs 14.3 ± 3.6 and 57.0 ± 7.1 vs 28.2 ± 5.5; 71.1 ± 5.1 vs 30.2 ± 1.7; $p < 0.01$; Fig. 2b, c & e, Table 2) in both patient categories while the levels of $CD4^+$ and $CD4^+CD28^+$ T cells were comparable (29.0 ± 1.3 vs 28.0 ± 1.7; 91.0 ± 1.2 vs 88.6 ± 1.6 and 32.4 ± 1.1 s 32.2 ± 1.6; 86.2 ± 3.1 vs 85.4 ± 3.8; $p > 0.1$, Fig. 2a, d & e). ANC controls were associated with higher levels of $CD4^+CD152^+$ T cells as compared to that of non-ANC controls (30.2 ± 1.7 vs 14.3 ± 3.6; $p < 0.001$). When we compared patients' groups, the $CD4^+$ T cells and sub-populations were not different ($p > 0.05$, Table 2).

3.3. Plasma cytokines

We observed significantly higher levels of all the assessed serum cytokines i.e. IL6, IL8, IL12 and TNFα in the non-ANC patients than in the non-ANC controls (3.3 ± 0.3 vs 15.5 ± 3.2; 3.7 ± 0.5 vs 105.9 ± 19.4; 8.4 ± 2.4 vs 31.2 ± 16.9 and 8.8 ± 0.9 vs 61.6 ± 10.6; $p < 0.05$; Fig. 3, Table 3). In the ANC controls, the levels of IL8 and TNFα were higher than in the non-ANC controls (12.5 ± 3.3 vs3.7 ± 0.5 and 23.8 ± 1.3 vs8.8 ± 0.9; Fig. 3b & d; $p < 0.05$) whereas IL6 and IL12 were comparable (Fig. 3a & c; $p > 0.9$). Comparison of ANC control and patient groups revealed lower levels of IL12 and TNFα in the patient category (6.2 ± 1.4 vs 3.1 ± 0.3 and 23.8 ± 1.3 vs 12.1 ± 2.8; $p < 0.05$; Fig. 3c & d). The ANC-patients exhibited lower levels of IL6, IL8, IL12 and TNFα when compared to the non-ANC patients (Fig. 3; $p < 0.001$; tale-3).

3.4. Gene expression in the PBMCs

We carried out gene expression analysis of selected genes from PBMCs representing different study populations. Overall, the degree of up-regulation ranged from 2.0 fold to 4.4 fold while none of the genes were down-regulated.

In the non-ANC patients, except for CD4, all the other genes examined (CD14, CD28, CD80, CD86, CD152 and HLA-DRA) were up-regulated (2.2 to 4.4-fold; Fig. 4). ANC controls exhibited higher expression of CD80 (4.0 fold) and HLA-DRA (2.6 fold) genes than in the non-ANC controls. Among the ANC subjects, the patient category exhibited increased expression of CD14, CD28, CD80, CD86, CD152 and HLA-DRA genes (2.0 to 4.0-fold), as compared to the controls.

4. Discussion

This study enumerates $CD14^+$ monocytes and $CD4^+$ T cells with their associated surface molecules in self-recovering hepatitis E patients. As circulating monocytes are primary targets for viruses [15–19], it is important to understand the activity pattern of surface molecules present on the $CD14^+$ monocytes in uncomplicated HEV infections that could form basis for comparisons with patients with fulminant outcome.

Monocytes and neutrophils are the primary phagocytes and reduction of these cells leads to increased susceptibility to microbial infections. The elevated frequency of $CD14^+$ monocytes in the ANC controls ($p < 0.001$, Fig. 1a & e) suggests heightened phagocytic barrier, a required immune alteration during healthy pregnancy [24]. 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 (Fig. 1 a & e, $p < 0.01$) providing evidence for the role of monocytes in recovery from HEV infection. In a recent report, Hepatitis E patients were shown to circulate higher $CD14^+$ monocytes [25]. The concomitant increase in the CD14 mRNA levels in non-ANC and ANC patients are noteworthy (Fig. 4). Importantly, though the monocyte frequency in control-ANC category was higher, HEV infection did not cause further significant increase. Role of monocytes in fulminant outcome of the disease during pregnancy needs to be undertaken.

Monocytes also play a central role in the innate response. In the non-ANC patients, we observed higher frequency of $CD14^+$ monocytes (and higher mRNA expression, Fig. 1a, g & 4) along with higher co-expression of TLR2 and TLR4. Earlier, we demonstrated a concomitant elevation of TLR4, TLR7 and TLR8 in the PBMCs of non-ANC patients at

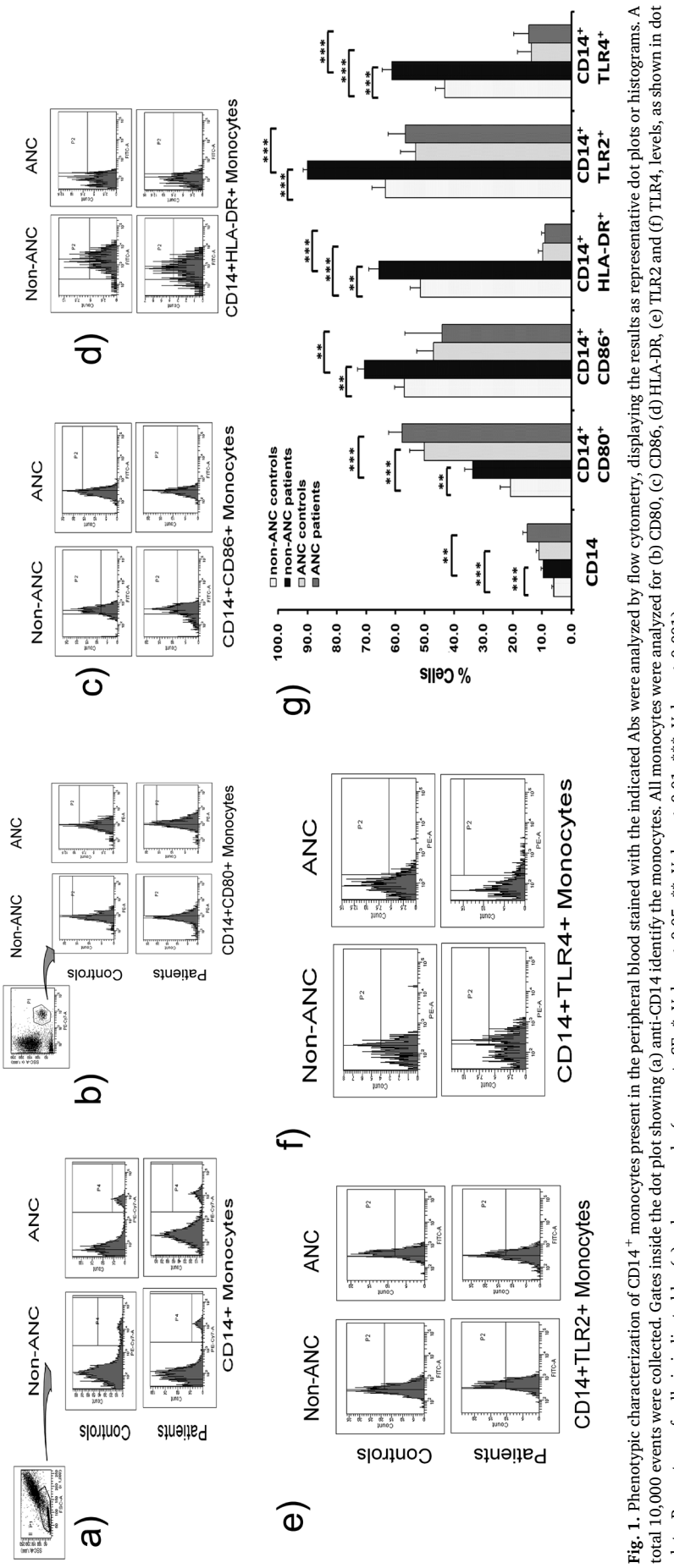

Fig. 1. Phenotypic characterization of $CD14^+$ monocytes present in the peripheral blood stained with the indicated Abs were analyzed by flow cytometry, displaying the results as representative dot plots or histograms. A total 10,000 events were collected. Gates inside the dot plot showing (a) anti-CD14 identify the monocytes. All monocytes were analyzed for (b) CD80, (c) CD86, (d) HLA-DR, (e) TLR2 and (f) TLR4, levels, as shown in dot plots. Percentage of cells is indicated by (g) column graphs (mean ± SE; *pValue < 0.05; **pValue < 0.01; ***pValue < 0.001).

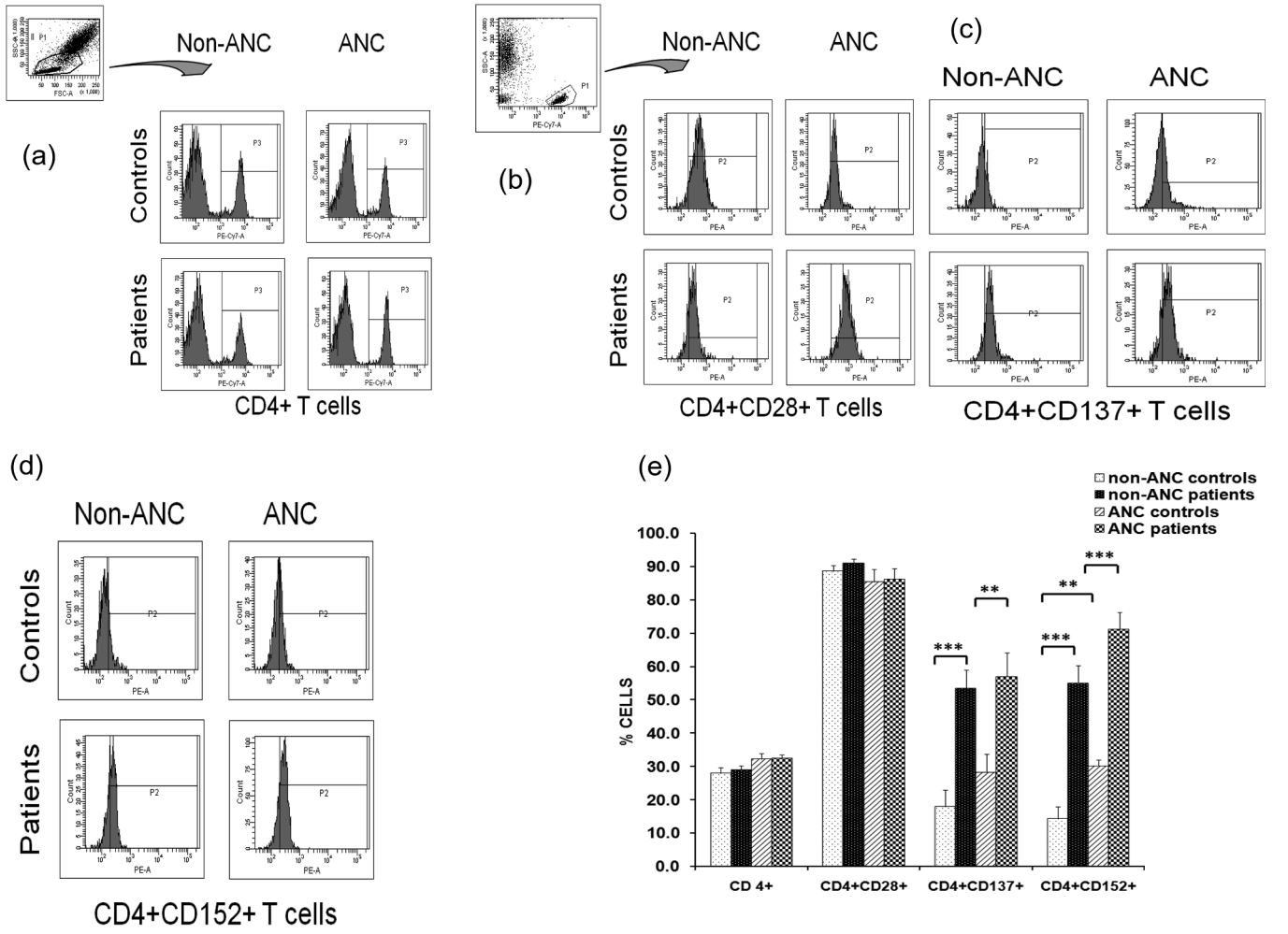

Fig. 2. (a) $CD4^+$ T cells were gated, as shown in dot plot and analyzed for (b) CD28, (c) CD137 and (d) CD152 levels as shown in histogram plots. A total 10,000 events of whole blood were collected and analyzed. Percentage of cells is indicated by (e) column graphs (mean ± se). (*pValue < 0.05; **pValue < 0.01; ***pValue < 0.001).

protein (by flow cytometry) and mRNA levels (TLDA), while a significant decline was noted in the ANC patients [26]. 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 TLR4 and TLR2 in the activation of $CD14^+$ monocytes. Similar observations were reported for Dengue and RSV viral infections [15–18].

Lower proportion of $TLR4^+$ monocytes ($p < 0.001$) in the ANC controls suggests impairment of TLR4 on monocytes during pregnancy. As compared to non-ANC patients, induction of TLR4 was not observed in the ANC patients. In fact, a significant reduction in the frequencies of $TLR2^+$ and $TLR4^+$ monocytes was seen in the ANC patients ($p < 0.001$ for both) as compared to the non-ANC patients. A significantly lower TLR mRNA levels in the ANC patients than in the non-ANC patients was shown earlier by us [26]. Taken together, these observations suggest altered activation of monocytes via TLR2 and TLR4 during hepatitis E in pregnancy.

We further analyzed the co-stimulatory markers such as CD80 and CD86 and HLA-DR on the $CD14^+$ monocytes. In the non-ANC patients, significantly higher expression of these co-stimulatory molecules at protein and mRNA levels suggests classical activation of the monocytes, antigen uptake and presentation during HEV infection. These results are in accordance with earlier findings of higher expression of DCs and associated activation markers during HEV infection [25,27]. The higher levels of inflammatory cytokines in the plasma of hepatitis E patients (Fig. 3) further support circulation of activated $CD14^+$ monocytes, an indication of inflammatory response during HEV infection.

ANC controls were characterized by increased and normal expression of the co-stimulatory molecules CD80 and CD86 respectively, and lower expression of HLA-DR on $CD14^+$ monocytes that remained comparable in the ANC patients suggesting altered activation of monocytes by HEV during pregnancy. However, when the patient categories were compared, the ANC-patients exhibited a significant reduction in the expression of CD86 and HLA-DR and increased frequency of CD80 indicating reduced antigen-presenting ability in these patients, probably due to the incomplete up-regulation of the co-stimulatory molecules. 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 [28]. These observations need to be confirmed by assessing HEV-specific immune responses in pregnant women with hepatitis E.

The next aim was to examine phenotypic activation of T helper cells during HEV infection. While confirming our earlier observations of the unaltered proportion of $CD4^+$ T cells in self-recovering non-ANC patients [20], we showed similar pattern in ANC patients and the corresponding controls. These patients had uneventful recovery. Importantly, a lowered $CD4^+$ T cell count was shown in ANC patients with fatal outcome [8].

We further examined the co-stimulatory molecules present on the $CD4^+$ T cells (Fig. 2). HEV infection among non-ANC and ANC

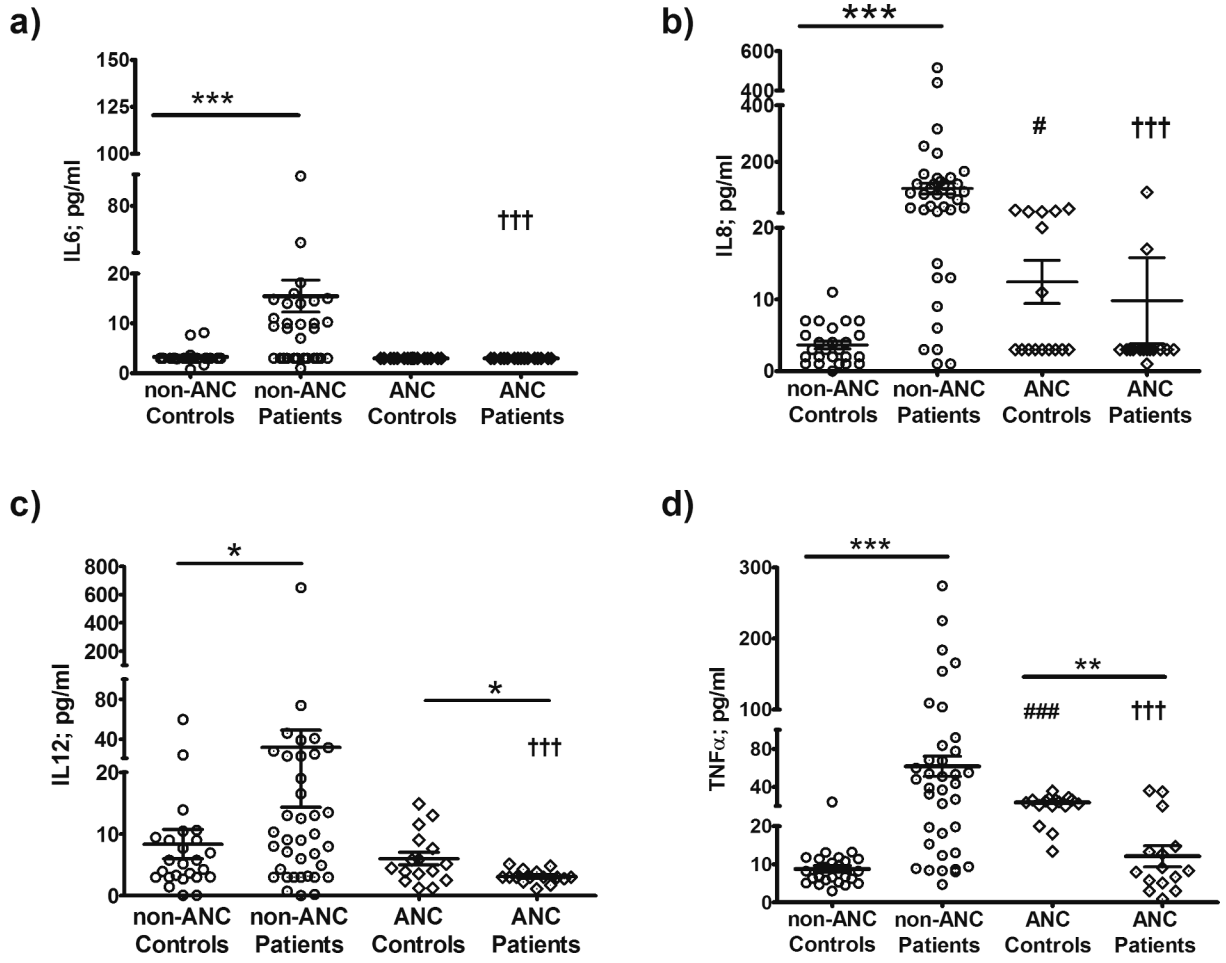

Fig. 3. Cytokine levels in plasma from different study groups. Levels are indicated by column graphs (mean ± se). (a) IL6, (b) IL8, (c) IL12 and (d) TNFα. (*pValue < 0.05; **pValue < 0.01; ***pValue < 0.001; # and † showing significance between non-ANC controls Vs ANC controls and non-ANC patients Vs ANC patients respectively).

categories did not alter $CD4^+CD28^+$ cells, though higher mRNA levels of CD28 were noted. CD137 co-stimulation has been shown to impact both $CD4^+$ and $CD8^+$ cell functions. CD137 is also expressed on the surface of Treg cells and plays crucial role in Treg function. The generation, survival and effector functions of CTLs (effector and memory) require CD137 expression [29–31]. However, our study was restricted to $CD4^+$ cells, CD137 expression on $CD8^+$ T cells in HEV infection remains to be investigated.

Cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) is considered to be critical for suppressive function of Tregs [32]. Our group demonstrated increased levels of CD152 on both regulatory and effector T cells [22,33] and showed that regulatory T cells in hepatitis E patients were functional and exhibit suppressive activity. The increased frequency of $CD4^+$ $CD137^+$ and $CD4^+152^+$ cells in both non-ANC and ANC patients observed during this study support the role of regulatory T cells in HEV infection in both the patient categories.

Among controls, the ANC controls expressed higher levels of $CD4^+152^+$ T cells while no difference was recorded in $CD4^+CD28^+$ and $CD4^+CD137^+$ T cells indicative of role of suppressive activity of regulatory T cells in healthy pregnancy [34]. Importantly, levels of all the T cell subtypes were comparable in ANC and non-ANC patients suggesting similar T cell response in both patient groups.

In accordance with the activated $CD14^+$ monocytes or/and activated T cells, we found higher levels of circulating inflammatory cytokines (IL12, TNFα, IL6 and IL8) in the non-ANC patients. Higher levels of IL8 and TNFα in the ANC controls suggest a weak inflammatory response during pregnancy. However, when ANC-controls were used for comparison, higher secretion of IL12 and TNFα by HEV infection was

Table 3
Levels of cytokines (pg/ml; Mean ± SE) in HEV patients and corresponding controls.

	non-ANC controls	non-ANC patients	ANC Controls	ANC Patients	*	**	#	†
IL6	3.3 ± 0.3	15.5 ± 3.2	3.2 ± 0.3	3.5 ± 0.5	0.0000	1.0000	1.0000	0.0002
IL8	3.7 ± 0.5	105.9 ± 19.4	12.5 ± 3.3	9.8 ± 5.9	0.0000	0.0569	0.0130	0.0000
IL12p70	8.4 ± 2.4	31.2 ± 16.9	6.2 ± 1.4	3.1 ± 0.3	0.0373	0.0288	0.9148	0.0002
TNFα	8.8 ± 0.9	61.6 ± 10.6	23.8 ± 1.3	12.1 ± 2.8	0.0000	0.0010	0.0000	0.0001

* non-ANC controls Vs non-ANC patients
** ANC controls Vs ANC patients
\# non-ANC controls Vs ANC controls
† non-ANC patients Vs ANC patients

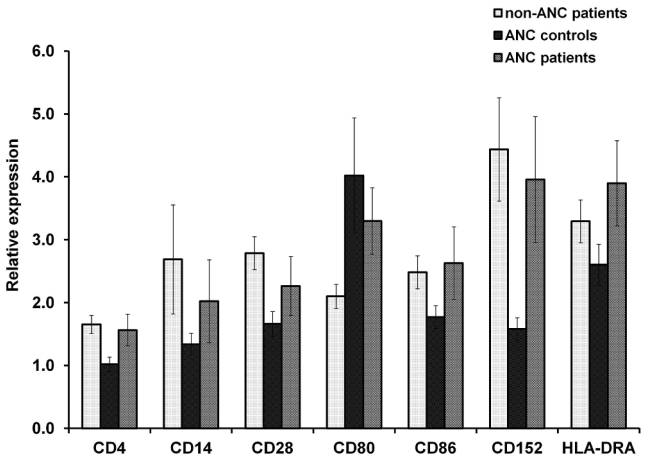

Fig. 4. Gene expression profiles of total PBMCs from the non-ANC and ANC study subjects were determined using TaqMan Low Density Array (TLDA). PBMCs from 16 non-ANC healthy individuals were taken as controls. The data presented are fold changes in the gene expression levels. Gene expression is indicated by column graphs (mean ± se; *pValue < 0.05).

apparent. On contrary, impaired response was evident in the ANC patients as judged by diminished levels of all the 4 cytokines when compared to the non-ANC patients. These results are suggestive of impaired monocytes-associated immune response in hepatitis E during pregnancy.

In summary, based on this cell enumeration study 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 patient groups. These observations need to be extended to fulminant hepatitis E during pregnancy with or without fatal outcome.

5. Financial disclosure

This study was funded by the Indian Council of Medical Research (ICMR), Ministry of health and family welfare, Government of India.

Declaration of Competing Interest

The authors declare they have no conflicts of interest.

Acknowledgements

The authors sincerely thank Director, National Institute of Virology, Pune for all the support. Excellent technical help by Mr Tilekar, Mr Jawalkar and Mr Shirish, statistical assistance by Mr. Walimbe are gratefully acknowledged. Financial assistance for this study was provided by the Indian Council of Medical Research, New Delhi and National Institute of Virology (NIV), Pune. RP Arya was the recipient of a fellowship from the University Grant commission, New Delhi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.06.011.

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