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Gene Expression Profiles of T cells from Hepatitis E Virus Infected Patients in Acute and Resolving Phase

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ABSTRACT

Background and Aims: Approximately 50% of acute viral hepatitis in young adults and in pregnant women is due to Hepatitis E virus (HEV) infection in developing countries. T cell mediated immune injury probably plays a key role in the pathogenesis of acute hepatitis illness. However, there is a paucity of data on the global gene expression programs activated on T cells, which are subsequently responsible for T cell recruitment to the liver and triggering of immune injury. **Patients and Methods:** We performed a flow cytometric analysis of T cells in individuals with acute hepatitis E (AVH-E; n=10), resolving phase of HEV (n=9) and 10 healthy controls (HC). Further transcriptional profiling analysis was performed using Affymetrix GeneChip DNA microarrays to identify the genes that were differentially expressed in AVH-E and HC. **Results:** Patients with AVH-E showed higher frequencies of CD8⁺ ($27 \pm 4\%$; $P=0.02$) and activated CD38⁺CD69⁺ T cells ($25\% \pm 3\%$; $P=0.04$) than in resolving phase patients ($20 \pm 2\%$ and $9.1 \pm 4\%$, respectively), who in turn exhibited higher CCR9 expression than cells from patients in active phase. The naïve T cell population (CD3⁺CD45RA⁺) was decreased upon HEV infection ($29 \pm 4\%$ in AVH-E vs. $53.1 \pm 3.2\%$ in HC; $P=0.05$), however, the CD11a high subpopulation within CD4⁺CD45RA⁺ cells was increased in both AVH-E (6.1%) and resolving phase (7.7%) patients. Gene ontology analysis suggested that during AVH-E infection there is in CD4⁺ T cells an activation of genes involved in pro-inflammatory responses. Additional RT-PCR analysis confirmed that in cells from AVH-E patients there is an increased expression of CCR5, CCR9, CXCR3, CXCR4, STAT1, IRF-9, IFN- α and TNF- α , together with a down-regulation of IL-2, SOCS3 and IL-10, respect to cells from resolving phase patients. **Conclusions:** Our findings suggest the involvement of a circulating CD45RA⁺ CD11a high population with CCR5 expression in the pathogenesis processes of AVH-E. The obtained results help to understand the underlying inflammatory process occurring in HEV infection, which can lead to either resolution or immunopathology.

Key Words: Acute Hepatitis E (AVH-E), CD4⁺ T cells, Microarray, Chemokine receptors, Peripheral blood mononuclear cells, Transcriptional profiling

Introduction:

Hepatitis E, a feco-orally transmitted virus, causes large-scale epidemics and sporadic cases of acute viral hepatitis in developing countries (1-3). However, recent outbreaks of acute infection caused by novel hepatitis E virus (HEV) strains have been described in USA, Japan and Europe (4-6). The HEV can also induce chronic hepatitis in immunosuppressed patients (7, 8). Approximately 50% of acute viral hepatitis in young adults are caused by HEV infection in developing countries, with fatality rates of more than 20% in pregnant women (9-11). Acute hepatitis E (AVH-E) infection in pregnant women is associated with higher maternal mortality rate and worse obstetric and fetal outcomes than any other type of viral hepatitis (9).

Following acute liver injury, the clinical manifestations and outcome of viral hepatitis are actually determined by the host immune response. Previous studies suggested that humoral immunity has neutralizing activity (12). However, its overall efficacy and role in protection from HEV infection remain unclear. In contrast with HBV or HCV infection, data on cellular immune responses in HEV infection are very limited (13-15). After entering through the oral route and inducing intestinal immunity, the HEV re-circulates through liver and arrives to peripheral blood.

It is unclear whether liver damage in HEV infection is caused by the virus itself or by host immune responses directed against infected cells (13). However, fragmentary *in vitro* data on PBMCs proliferation and HEV-specific T cells producing Th2 cytokines argue for a role of CD4⁺T lymphocytes in HEV infection and liver immune-mediated pathogenesis (16, 17). In addition, it seems that the outcome of HEV infection will depend on both the T cell subsets and antigen non-specific inflammatory cells recruited under particular conditions to the liver. Being HEV self resolving, the phenotypic expression of memory makers on the recruited effector T cells were not studied. This process is mainly guided by the expression of specific chemokine receptors (18). Thus, it would be important to address the activation status and expression of chemokine receptors of T cells from patients at different phases of

the infection. However, up to now there is no comparative evaluation of expression profiles of immune cells from the periphery in patients during the acute and resolving phases of HEV infection with respect to HC.

Thus, to gain further insights on the pathogenesis of HEV infections we have analysed the overall gene expression profiles in CD4+T cells recovered during the acute phase of illness in comparison to HC.

METHODS:

Patients:

Acute HEV infection (AVH-E; n=10): Patients with diagnosed acute hepatitis, having jaundice, fever, vomits, raised AST and ALT, > 10x ULN, serologically positive for IgM anti-HEV and positive for HEV RNA within four week of the initial symptoms, were included.

Acute HEV resolving phase patients (n=9):

Patients with clinically diagnosed icteric viral hepatitis, at least 4 weeks after the icteric illness and within 12 week of the follow up visit, in whom the disease clinically subsided.

Healthy controls (HC; n=10): The recruited healthy controls had no previous history or current evidence of any liver disease, with normal serum values of transaminases.

All the enrolled patients and HC tested negative by enzyme immunoassays (EIAs) for hepatitis B surface antigen, IgM anti-hepatitis A virus antibody, anti-HCV and anti-HIV antibody (M/S Ranbaxy laboratories, India).

Exclusion criteria: Patients with past history of chronic liver disease, clinical or radiological evidence of chronic liver disease, regular alcohol consumption (> 40g/day for the past 5 years), diabetes, severe systemic illness, pregnancy, or co-infection with HIV, HBV or HCV were excluded from this study.

From the selected patients and HC, peripheral blood was collected in an EDTA coated tube. Plasma samples were stored at -20°C. IgM anti-HEV antibodies were detected with commercial EIA kits (Genelabs Diagnostics/MP Biomedicals Asia Pacific,

Singapore). The biochemical assessment was done according to the study protocol. An institutional ethics committee approved the study protocol and all study subjects provided informed consent.

HEV Quantification using TaqMan-Based Real-Time RT-PCR:

HEV RNA was extracted from samples using QIAamp viral RNA mini kit (Qiagen, Germany). HEV-RNA load (copies/ml) was determined by Taqman RTPCR using primers and probe corresponding to HEV ORF1 (7300 Real Time PCR system, Applied Biosystems, USA). Positive control was generated from a 1067-bp fragment of HEV genome (4,632–5,698 nt), which was PCR amplified and cloned into pGEM-T Easy. Standard curve showed linear relationship ($r^2 = 0.99$) from 10 to 10^{10} RNA copies/reaction. The sensitivity of the assay was 100 copies/ml.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque density gradient centrifugation from freshly collected blood samples at baseline and on every follow up from the groups and were stored in freezing media containing 10% DMSO and 90% FCS in liquid nitrogen until used.

Flow cytometric analysis

T cell subsets from PBMCs were characterized by flow cytometry using anti-CD3-PeCy7, anti-CD8-FITC, anti-CD45RA-FITC, Anti-CD11a-PE, anti-CXCR3-FITC, anti-CCR1-PE, anti-CCR3-PE, anti-CCR5-PE, anti-CCR6-PE, anti-CCR9-PE, anti-CD38-FITC and anti-CD69-PE (BD Pharmingen, USA). Cells were stained for 20 min at room temperature and then washed twice with PBS and fixed using 0.1% paraformaldehyde. Single color compensation was performed prior to acquisition of samples. More than 30,000 cells were acquired for flow cytometric analyses using a FACS Calibur and the results were analyzed using the Flow-Jo software.

DNA microarray hybridization and analysis: Quality and integrity of the isolated total RNA was controlled by running all samples on an Agilent Technologies 2100 Bioanalyzer (Agilent

Technologies; Waldbronn, Germany). For biotin-labeled target synthesis reactions were performed using standard protocols supplied by the manufacturer (Affymetrix; Santa Clara, CA).

The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 10 µg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre), as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix GeneChip HG-U133 Plus 2.0 for 16 h. After hybridization the GeneChips were washed, stained with SA-PE and read using an Affymetrix GeneChip fluidic station and the GCS3000 scanner.

Microarray data analysis

Analysis of microarray data was performed using Affymetrix GCOS 1.2. For normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of GCOS 1.2. The pair wise comparison of signal intensities was conducted by the MAS5 statistical software (Affymetrix, Santa Clara, CA). Differential expression of genes was determined by a fold change of two and statistical significance for expression change calculated by the MAS5 algorithm. Gene ontology analysis was performed on differentially expressed genes by using the Gene spring GX10 software (Agilent Technologies; Waldbronn, Germany).

The indicated P-value for individual GO terms, also known as the enrichment score, signifies the relative importance or significance of the GO term among the genes in the selection as compared to the genes in the whole dataset.

Quantitative real-time PCR analysis:

Total RNA was isolated from PBMC's by using the Qiagen RNA isolation kit. cDNA was prepared using random hexamers and MuLV reverse transcriptase enzyme. For the validation of the results emerging from the array studies for selected genes quantitative

real-time RT-PCR was performed in the Roche detection system using the Syber Green kit and forward and reverse primers for

CCR1: CTCTTCCTGTTACGCTTCC and GCCTGAAACAGCTTCCACTC,

CCR3: GTCATCACCAGCATCGTCAC and TCATGCAGCAGTGGGAGTAG,

CCR5: GGGATAGCACTGAGCAAAGC and GTCATCACCAGCATCGTCAC,

CCR6: CTGCAGTTCGAAGTCATC and GTCACACCACCATAATGTTG,

CCR9: AGCGATGAGAGCACCAAAGT and TGGCTTGTATCAGGGTGTGA,

IFN-A: GCCCAGAGAGCAGCTTGACT and CCTCGCCCTTTGTTTACTG,

TNF-A: TCTCGAACCCCAAGTGACA and AGCTGCCCTCAGCTTGA,

IL-2: CACAAACAGTGACCTACTTCAAGT and CAAAATCATCTGTAAATCCAGCAGTAA,

IL-4: AACGTACTCTGGTTGGCTTC and TGCCTCCAAGAACACAAGT,

IL-10: CCGCCTCAGCCTCCCAAAGT and CCCTAACCTCATTCCCCAACCAC,

CXCR3: GCGAAGAAAGCCAGGATGAGGAT and GCCTTATCCTGCCTGGTATTGTC,

STAT1: CCTGTTAATGAACACCATGCTCATA and CGATGCAGGCGATTTGCT,

SOCS3: ACTTCGGGAATGCTGAACTAATG and GAGCAAACAAGTTCCGTTGGA,

IRF-9: GATTCCTTGGAACATGCAG and CTTCCAGACAGCTGGACCTC,

FOXP3: ACACCACCCACCACCGCCACT and TCGGATGATGCCACAGATGAAGC.

The primer pairs were designed using the Primer express software. All RT-PCR reactions were performed in triplicates. Amplification of β -actin was used as the endogenous control for normalization. Relative quantification of each gene was analyzed by calculating of each sample Ct value.

Statistical analysis

All clinical parameters; serum bilirubin, albumin, ALT and AST were expressed as median with range. Non-parametric Mann-Whitney U test was used to calculate the P values (<0.05 were considered as significant).

RESULTS:

Clinical and virological characteristics of subjects affected by AVH-E:

The clinical characteristics of both the HEV infected patients in acute or resolving phase and the HC enrolled in this study are shown in Table 1. A median value of 195 copies/ml was observed for HEV RNA (range 100-650 copies/ml). There were no significant differences in the age, sex and albumin levels of HC and HEV infected patients. AST and ALT levels were significantly higher ($P \geq 0.05$) in AVH-E patients than in those in the resolving phase and HC. However, the serum bilirubin levels did not show a significant decrease in resolving patients.

Higher frequencies of CD8+ T cells and CD3+ CD38+ CD69+ T cells are observed in patients in AVH-E phase than in those from resolving phase patients:

In AVH-E infection, patients had significantly higher frequencies of CD8+ T cells than in controls (AVH-E [0-4 wk] $27 \pm 4\%$ vs. HC $16 \pm 3.5\%$; $P=0.02$). These values were normalized during the resolving phase of infection ($20 \pm 2\%$, $P=NS$). There was no significant difference in the frequencies of CD4+ T cells during the acute and resolving phase of HEV infection with respect to those of HC (HC $67.6 \pm 6\%$ vs. AVH-E [0-4 wk] $71 \pm 4\%$ vs. resolving HEV [<4 wk] $79 \pm 8\%$; $P = NS$) (Fig.1A, B). In contrast, a significant difference was observed in the percentages of CD3+ activated T cells (CD38+CD69+) in AVH-E patients respect to HC and resolving phase patients (HC $1.1 \pm 0.5\%$ vs. AVH-E $25 \pm 3\%$ [$P=0.04$] vs. resolving HEV $9.1 \pm 4\%$ [$P=NS$]; Fig. 1C).

A decreased pool of naïve T cells is observed during HEV infection:

HEV is a self limiting infection, which results in the stimulation of long term memory. It was observed that a significant reduction in the population of naïve CD3+ CD45RA+ T cells occurs during AVH-E with respect to the basal values in HC and resolving phase patients (HC $53.1 \pm 3.2\%$ vs. AVH-E [0-4 wk] $29 \pm 4\%$ $P=0.04$ vs. resolving HEV [<4 wk] $41 \pm 3.5\%$; Fig.2A).

Differential expression of the CD11a integrin and chemokine receptors on CD45RA+ naïve T cells:

When the phenotypic profiles of circulating CD4+CD45RA+ and CD8+CD45RA+ cells were analyzed for the expression of the CD11a integrin (LFA-1), a biphasic distribution of CD11a high and CD11a low subpopulations was observed. Both in acute and resolving HEV patients, CD4+CD45RA+ T cells showed a significantly higher subpopulation of CD11a high cells than HC (HC 0.6% vs. AVH-E [0-4 wk] 7.7% vs. resolving HEV [<4 wk] 6.1%; $P=0.05$) (Fig.2B). In contrast, there was no significant difference in the CD11a high population in CD8+CD45RA+T cells (HC 7% vs. AVH-E [0-4 wk] 10% vs. resolving HEV [<4 wk] 11%; $P=NS$). On the other hand, in both CD4 and CD8+T cells, the CD11a low population was significantly skewed in the acute phase. In AVH-E patients, in CD4+T cells the CD11a low subpopulation was 6% vs. 18% in HC and 23.2% in resolving phase patients, whereas in CD8+ T cells the CD11a low subpopulation was 5% vs. 37% in HC and 34% in resolving phase patients; Fig.2C).

Interestingly, in both acute and resolving phase patients naïve T cells co-express the chemoattractant receptors CCR5, CCR9 and CXCR3. CCR5 was over expressed during the acute and resolving phases ($P=0.002$), whereas the expression of CXCR3 was significantly higher in resolving phase ($P=0.04$) and CCR9 expression on CD3+T cells of resolving phase patients ($P=0.04$; Fig. 3).

Global gene expression patterns in HC and AVH-E patients:

To identify differentially expressed genes in CD4+ T cells from AVH-E patients and HC signal log ratios were calculated and their significance for expressional change determined. Out of 42,000 genes present on the human Affymetrix GeneChip, 363 genes were differentially expressed in AVH-E infected patients as compared to HC (i.e. 346 and 17 genes were up- and down-regulated, respectively). For a more detailed view of the functional consequences for CD4+ T cells resulting from HEV infection, we mapped these gene entities to gene ontology (GO) classes. We found for most of the GO classes with no significant enrichment. One third to two thirds of the gene entities were mapped to the

major GO classes (cellular process: 166 genes, 22%; biological processes: 111 genes, 14.53%; metabolic processes: 101 genes 13.2%). However, no GO class was over represented by the selected gene entity list except for the GO class immune system processes (32 genes, 4.2%, corrected P value: 0.1633; see Fig. 4). Here, we observed an accumulation of immune genes as compared to the number of genes that can be expected by chance. Although this observation is not unexpected as response of an immune cell to a viral infection, it might also indicate HEV specific modulation of the CD4+ T cell transcriptome favoring viral persistence.

Quantitative RT- PCR for cytokines, chemokine receptors, and IFN pathway:

The results obtained by flow cytometry and transcriptional profiling were further confirmed for selected genes by performing mRNA expression analysis by RT-PCR in samples from acute or resolving HEV infected patients and HC. To this end, we have independently analyzed the expression of CCR1, CCR3, CCR5, CCR6, CCR9, CXCR3, CXCR4, IL-2, IL-4, IL-10 and IFN pathway related genes, such as IRF-9, STAT1, SOCS3, IFN- α and TNF- α (Fig.5-7). The mRNA expression levels of CCR9 and CCR5 was higher in AVH-E and resolving phase patients than in HC, however, the expression of CXCR3 and CXCR4 was higher only in cells from resolving phase patients (Fig. 5). On the other hand, the expression of the cytokines IL-4, IFN- α and TNF- α was higher in samples from AVH-E than in cells from HC or resolving phase patients, whereas IL-2 expression was decreased in both acute and resolving phase and IL-10 was reduced only in resolving phase (Fig. 6). Concerning type 1 interferon signaling pathway molecules, SOCS3 expression was down-regulated, whereas the expression of STAT1 and IRF-9 was higher during the acute phase of infection (Fig.7).

DISCUSSION:

In present study, we investigated the expression of tissue-specific homing receptors on effector/memory lymphocytes after HEV antigen encounter. During infection, antigen specific lymphocytes get activated, thereby leading to a differentiation of naïve cells into

effector and memory cells, which display appropriate homing receptors on their surface (20, 21). We hypothesized that the expression of tissue-specific homing receptors on the surface of effector/memory lymphocytes and their temporal recirculation in blood after antigen encounter will allow us to use the peripheral cells to address the contribution of T cells to HEV pathogenesis, since considering the current state –of-the- art in the management of hepatitis patients, liver biopsies cannot be proposed as a routine intervention due to ethic constraints.

In our study we have observed in AVH-E an increase in the proportion of CD8+ T cells, but no obvious changes in the CD4+ T cell compartment in the periphery. However, there was an increased expression of the CD11a integrin in naïve CD45RA+ T cells, as well as over expression of CCR5 and CCR9 during AVH-E infection. The presence of an expanded CD45RA+CD11a high subpopulation during infection suggests enhanced recruitment of these cells from periphery to the target tissue during the early phase of infection. In fact, chemokines and their receptors, as well as other activation and adhesion molecules, are critical for the recruitment from the periphery, trafficking and homing of effector immune cells to sites of inflammation (22-24). Higher CCR9 expression on T cells identifies their homing character from gut to liver (22, 25).

Thus, the overall picture of expression of tissue-specific homing receptors (i.e. CCR5, CXCR3 and CXCR4) on the surface of effector/memory peripheral lymphocytes emerging from our studies suggests a targeting of these cells for specific homing to the liver. In fact, a large proportion of CD45RO+, CD69+, CD27+ and CD11a+ T cells expressing CCR5 and CXCR3 were detected in hepatocellular carcinoma (26). CCR5 also directs the homing of CD4+CD25+ nTreg cells and activated T cells to the site of infection (27). Viral replication also results in the induction of cytokines, which further amplify the infiltration of mononuclear leukocytes. We have observed an expansion of CD38+CD69+ T cells in the acute phase respect to samples obtained from the resolving phase of infection. This was associated with an increased in mRNA expression for IFN-gamma, TNF- α and IL-4, and slight down-regulation of IL-2 and IL-10 in CD4+ T cells. This could reflect an increment in

CD3+CD38+CD69+ T cells, which produce type I (IFN- γ , TNF α) and type II (IL-4) cytokines in AVH-E.

Interplay between the effects from IL-10 and IL-4 can then determine the course of progression of hepatic inflammation (28). The local release of cytokines may also affect leukocyte infiltration, activation, and up-regulation of vascular adhesion molecules. In the past, studies have compared the cytokine milieu of AVH-E and HEV induced fulminant hepatic failure (FHF). Increased in both IFN-gamma levels in supernatants of HEV ORF2-stimulated PBMCs and IFN-gamma mRNA transcripts in cells were detected in AVH-E (13, 16). However, viral FHF is accompanied by local and systemic increases in IL-1, TNF- α and IL-6. As compared to AVH, significantly higher levels of both Th1 (IFN-gamma, IL-2 and TNF-alpha) and Th2 (IL-10) cytokines were recorded in FHF patients (16). The frequency of low IL-10-producing haplotypes was also higher in FHF patients respect to controls (28).

In this study we also found a high expression of STAT1 and IRF-9 and down-regulation of SOCS3 during the acute phase, which returns to basal levels during the resolving phase. Increased IFN- γ secretion and effective IFN signaling inhibits the viral replication with a functional Jak-Stat-IRF-9 pathway (29). Binding of IFN- α/β to the IFN receptor stimulates in turn the phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 associate with IRF-9 to form the transcription factor ISGF3, which re-localizes to the nucleus and stimulates the transcription of ISGs.

CONCLUSIONS:

Our data suggest that there is an activation of CD38+CD69+ T cells in the peripheral blood of HEV-infected patients during the acute phase. There are distinct patterns of expression for integrins, homing receptors and cytokines in cells obtained from patients in the acute and resolving phases. Our findings suggest the involvement of a circulating CD45RA+ CD11a high CCR5+ sub-population in the pathogenesis process of HEV infection. However, the interpretation of our results should be cautious, since they are limited to cells obtained from peripheral blood, which might not fully reflect what occurs in the local microenvironment of the liver (e.g. the high proportion of cytokine producing NKT cells may

lead to a different cytokine niche). Thus, our study emphasizes the need for in-depth immunological studies in HEV infected pregnant women and FHF, for whom liver tissue can be obtained.

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Figure Legends

Figure 1. Increased frequencies of CD8+T and CD3+CD38+CD69+ T cells in HEV infected patients.

A. Percentages of CD3+CD4+T cells in PBMCs of HC and patients during acute and resolving infection (P=NS). **B.** Percentage of CD3+CD8+T cells in PBMCs of HC ($16 \pm 3.5\%$) and patients during the acute phase ($27 \pm 4\%$ P=0.02) and resolving phase ($20 \pm 2\%$, P=NS) of infection. **C.** Percentage of CD3+CD38+CD69+ cells in PBMCs of HC $1.1 \pm 0.5\%$ vs. AVH-E $25 \pm 3\%$ [P=0.04] vs. AVH-E resolving $9.1 \pm 4\%$ [P=NS]).

Figure 2. Differential Expression of integrins in CD4+ and CD8+ T cell populations.

A. Significantly lower percentages of CD45RA+ naïve T cells were observed in the acute phase of infection (29%) than in HC (53%, P=0.04). **B-C.** Two distinct sub-populations of CD11a high and CD11a low CD45RA+ T cells are observed. **B.** In HC CD4+CD45RA+T cells show lower expression of CD11a high (0.6%) than in acute and resolving phase of infection (AVH-E 7.7% and resolving phase 6.1%). **C.** The CD11alow subpopulation represents 18 and 23.2% of the CD4+CD45RA+ cells in samples from HC and resolving phase patients, respectively, whereas only 6% in AVH-E patients. CD11alow cells represent 37 and 34% of the CD8+CD45RA+ T cells in HC and resolving phase patients, respectively, and 5% in AVH-E patients.

Figure 3. Increased Co-expression of chemokine receptors on CD3+ CD45RA+ T cells in AVH-E and resolving HEV phase patients.

Representative dot plots show the expression of CCR5 and CXCR3 on CD3+CD45RA and CCR9 on CD3+T cells from HC, AVH-E and resolving HEV infected patients, similar results were obtained in all the evaluated patients. Lower expression of CCR5 and CXCR3 on CD3+ CD45RA+ T was detected in HC, whereas a significant increase of CCR5 was observed in the AVH-E phase (P=0.002). The number of cells expressing CCR9 was significantly increased on lymphocytes in resolving HEV infection (P =0.04).

Figure 4. Global gene expression patterns in HC and AVH-E patients.

Analysis of microarray data was performed using Affymetrix GCOS 1.2 and data Mining Tool 3.1 software package. For normalization all array experiments were scaled to a target intensity of 150 using default values of GCOS 1.2. Gene Ontology (GO) terms were used to identify the major functional classes affected by the expression signatures obtained from AVH-E and HC expression datasets.

Figure 5. Quantitative Real Times PCR for chemokine receptors.

Figure depict relative expression levels, as ratios of normalized mean gene expression of infected patients were compared to healthy controls. The expression levels of chemokine receptors CCR1, CCR3, CCR5, CCR9, CXCR3 and CXCR4, which are known to play an important role in cell trafficking were analyzed.

Figure 6. Quantitative Real Times PCR for the expression levels of cytokines like IL-2, IL-4, IL-10, and TNF- α and IFN which are known to play an important role in progression of hepatic inflammation.

Figure 7. Quantitative Real Times PCR for the end molecules of Type 1 Interferon signaling pathway The expression levels of STAT1, SOCS3 and IRF-9, upregulation of these molecules effectively inhibit the replication of virus.

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Fig 7

Table 1. Clinical and virological characteristics of patients and healthy controls enrolled in the study

Parameters	Acute HEV (N=10)	Acute HEV Resolving Phase (N=9)	Healthy Controls (HC) (N=10)	P values
Age (Yr.) Mean± SD	25.2 ± 9.9	26.3 ± 7.5	29 ± 5	NS
Sex M : F	7: 3	4 : 5	6 : 4	NS
Serum Bilirubin (mg/dl) Median [range]	6.9 [2.6-18.5]	7.8 [0.4-19.1]	0.3 [0.1- 0.7]	Acute HEV vs. HC (P=0.02) Acute HEV vs. Resolving Phase (P=NS)
Albumin in g/dl Median [range]	4.15 [3.4- 4.7]	4.5 [3.1-7.5]	4 [3.5-4.2]	NS
AST (IU/L) Median [range]	250.5 [75-4010]	49.5 [15-100]	33 [30-35]	Acute HEV vs. HC (P=0.00) Acute HEV vs. Resolving phase (P=0.03)
ALT (IU/L)	604 (163-5267)	51 (10-71)		Acute HEV vs. HC

Median [range]			30 (30-34)	(P=0.00) Acute HEV vs. Resolving phase (P=0.00)
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