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TGF-β_1 and contact mediated suppression by $CD4^+CD25^+CD127^-$ T regulatory cells of patients with self-limiting hepatitis E

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ABSTRACT

Background and aim: Literature on the role of Regulatory T cells (Tregs) in acute viral infections is limited. Having established that the Tregs in self-limiting hepatitis E infection are elevated and functional, this study has focused on characterizing the specificity, phenotypes and identifying the molecules or factors responsible for enhancement of Treg cells and abrogation of Treg-mediated suppression in hepatitis E.

Methods: HEV rORF2p specific (a) Treg frequency, subset analysis and expression of surface and intracellular markers on Tregs and CFSE based functional analysis by flow cytometry (b) key cytokines quantification by multiplex (c) suppressive functional assay in the presence of anti-TGF-β_1 or anti-IL-10 or both antibodies or Transwell insert or in combination were performed on samples from 58 acute patients (AVH-E), 45 recovered individuals from hepatitis E and 55 controls.

Results: In AVH-E, the increased frequencies of Tregs and Teff cells were HEV rORF2p specific and Treg cells were of effector memory phenotype. Higher expressions of HEV rORF2p stimulated CTLA-4, GITR, PD1L, CD103, CD39, TLR2 and TGF-β_1 molecules on Tregs of AVH-E were observed. Tregs produced TGF-β_1 and inhibited the secretion of IFN-γ. Transwell insert and cytokines blocking assays indicated Tregs mediated suppression in AVH-E patients is majorly TGF-β_1 mediated and partly cell-cell contact mediated.

Conclusion: Overall, we have identified beneficial involvement of HEV specific, functional Tregs and TGF-β_1 as the regulatory molecule responsible for enhancement of Tregs in self-limiting HEV infection. Therefore, use of TGF-β_1 as a possible supplement for boosting Treg response in recovery from severe hepatitis E needs evaluation.

1. Introduction

Hepatitis E virus (HEV) is the major cause of self-limiting acute viral hepatitis in several developing countries, including India [1]. Although most people infected with hepatitis E virus take an asymptomatic course, the disease ranges from asymptomatic infection to self-limiting acute viral hepatitis (AVH-E) and a few patients may develop severe hepatitis that can progress to fulminant hepatic failure (FHF-E). In addition, cases of chronic hepatitis E rapidly progressing to cirrhosis in organ transplant recipients have been reported [2]. FHF-E due to hepatitis E has a worse outcome in elderly, pregnant women, and patients with underlying chronic liver disease [3]. The pathogenesis of hepatitis E appears to be substantially immune mediated [4]. Acute infection usually requires no treatment, a sole report has indicated ribavirin as an effective therapeutic agent for FHF-E and treatment of chronic hepatitis E with ribavirin monotherapy is recently published [5,6]. Now that a hepatitis E vaccine is available in the market, it is of heightened importance to understand and identify the key molecules regulating the immune response towards recovery.

Literature suggests a definitive role of T cells in the immunopathogenesis of HEV infection [7]. Reports of HEV-specific T cells

Abbreviations: Tregs, regulatory T cells; HEV, hepatitis E virus; rORF2p, recombinant open reading frame 2 protein; CFSE, carboxyfluorescein succinimidyl ester; TGF-β, transforming growth factor beta 1; IL10, interleukin 10; AVH-E, acute viral hepatitis E; Teff, T effector cells; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; GITR, glucocorticoid-induced TNFR family related gene; PD1L, programme death 1 ligand; TLR, Toll like receptor; FHF-E, fulminant hepatic failure; ALT, alanine transaminase; PBMCs, peripheral blood mononuclear cells; HCV, hepatitis C virus; HBV, hepatitis B virus.

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producing Th2 cytokine have further indicated role of CD4+ T lymphocytes in HEV infection [8,9]. Regulatory role of $CD4^+$ T lymphocytes in HEV infection and associated pathogenesis has been established [10]. Expansions of naive $CD4^+$ T cells in AVH-E patients have been attributed to the NKT or Treg cell populations [11]. We have shown higher frequencies of Tregs, T effector cells and Treg associated key conventional molecule expressions in self-limiting HEV infection and have thus established the involvement of Tregs in hepatitis E [12,13]. Further, we reported that Tregs in AVH-E patients has more suppressive activity than the recovered and control individuals and the Tregs might be modulating the disease course by controlling the inflammatory responses [12,13]. Absence of Tregs in liver of fulminant hepatitis E patients has been related with disease severity [14].

Tregs are classically identified by forkhead box transcription factor P3 (FoxP3), a key regulator in the development and function of the Treg population [15,16]. Due to the intracellular location of FoxP3, this marker cannot be utilised to isolate Tregs, essentially required for functional studies. Recent studies have demonstrated an inverse relationship between the surface expression of the interleukin 7 receptor (CD127) and FoxP3, thus allowing the use of this marker to assess both the level and functionality of Tregs [17,18]. It has been established that Tregs can suppress Teff cell proliferation and immune responses by means of cell to cell contact [19–21], and cytokine dependent mechanisms [22,23].

Having established that the Tregs are elevated, functional, an inverse correlation of Teff cell proliferation with plasma TGF-β_1 levels in self-limiting hepatitis E patients [24], this study has focused on characterizing the specificity, phenotypes and identifying the molecules or factors responsible for enhancement of Tregs or abrogation of Treg-mediated suppression in hepatitis E.

2. Materials and method

2.1. Patients

One hundred and fifty-eight individuals from Maharashtra, India, including 58 AVH-E patients, 45 recovered individuals from hepatitis E and 55 healthy controls (HC) were enrolled during August 2013-March 2014. Acute hepatitis E patients were from the hepatitis outbreak and none of the patient was admitted to a hospital or clinic. The controls were the accompanying persons with the patients and were apparently healthy with no other illness. The diagnosis of hepatitis E was based on the presence of IgM antibodies to hepatitis E virus (IgM anti-HEV) as detected by an in house developed ELISA [25]. The patients were classified as AVH-E based on the standard clinical and biochemical criteria [26]. Briefly, patients presenting with icterus, dark-coloured urine, elevated alanine aminotransferase (ALT) (normal level, 4–40 IU/L elevated level, >2.5 times to normal level) and/or bilirubin levels (>1 mg/mL) in the serum and/or presence of bile salts and pigments in the urine were considered to have acute hepatitis.

The recovered individuals had a previous history of acute hepatitis E. They had normalized ALT levels, positive for anti-HEV IgG antibody, and were negative/positive for serum anti-HEV IgM antibody. Control samples were collected from the same epidemiological condition as patients and were naive to HEV infection. The age of the controls were matched with AVH-E and recovered groups and varied within 5 years and were negative for IgM/IgG anti-HEV antibodies. All study subjects were negative for HBsAg, anti-HCV and IgM anti-HAV antibodies.

The study population characteristics are shown in Table 1. Among the total study population (n = 158), (1) HEV rORF2p specific frequency of Tregs were assessed in 24 AVH-E patients, 15 recovered and 30 controls (2) HEV rORF2p specific frequencies of Treg subsets were analyzed in 24 AVH-E patients, 15 recovered and 10 controls (3) HEV rORF2p specific functional assay and key cytokines were assessed in 6 AVH-E, 6 recovered and 6 controls and (4) Mechanism of Treg suppression assays were done in 6 AVH-E, 6 recovered and 4 controls. The study subject used for different assay was mentioned in figure legends. None of the patient was having any past history of chronic liver disease, severe systemic illness at the time of sampling. This study was approved by the *"Institutional Ethics Committee for Research on Humans"* as per the guidelines of Indian Council of Medical Research, India. Informed written consent was obtained from all the participants.

Table 1
Patients and control subjects characteristics.

Parameters	AVH-E	Recovered	Controls
Study population (no.)	58	45	55
Age (Years)	28.18 ± 10.04	30.95 ± 14.41	27.80 ± 3.39
Sex ratio (M:F) in percentage	37:21 (63.7:36.2)	28:17 (62.2:37.7)	32:23 (58.2:41.8)
ALT (IU/L)	409.60 ± 174.78$^{\dagger *}$	28.45 ± 8.04	24.20 ± 6.56
Viral load (copies/ml)	$6.9 \times 10^3 \pm 2.9 \times 10^3$	ND	NA
POD	10.96 ± 5.07	84.75 ± 6.29	NA

Data are presented as mean ± SD. $^{\dagger}p \leqslant 0.05$ comparing AVH-E and recovered individuals, $^{*}p \leqslant 0.05$ comparing control and AVH-E, and $^{\#}p \leqslant 0.05$ comparing AVH-E and recovered individuals. P-values were calculated using Mann-Whitney *U*-test and one-way ANOVA, followed by Tukey's test. Abbreviations: M, male; F, female; ND, Not detected; NA, Not applicable, ALT, Alanine transaminase; POD, Post onset days of illness.

2.2. Serological and molecular testing

Samples were screened for IgM antibodies against hepatitis A virus (anti-HAV IgM; Hepavase A-96, General Biologicals Corp, Taiwan), hepatitis B surface antigen (HBsAg; Surase B-96, General Biologicals), IgM antibodies against hepatitis B core antigen (anti-HBcIgM; Anticorase B-96, General Biologicals), antibodies against hepatitis C (anti-HCV; Ortho HCV 3.0, Ortho Clinical Diagnostics, USA), antibodies against HIV-1 (INSTI^{TM} HIV-1 antibody Test Kit, Biological Laboratories Inc, Richmond, British Columbia, Canada), anti-HEV IgM and IgG antibodies by an in house developed ELISA [25] and for serum alanine amino transferase levels (ALT; Span Diagnostics, India). Plasma HEV viral load was determined by Taqman reverse transcription polymerase chain reaction as previously reported [26].

2.3. Recombinant ORF2 protein preparation

Recombinant ORF2 antigen was expressed and purified as described previously [27,28].

2.4. Isolation of PBMCs and purification of $CD4^+$ T cell subpopulation

Peripheral blood mononuclear cells (PBMCs) were isolated, washed in RPMI-1640 medium (Life Technologies, USA), counted in the presence of a trypan blue dye and the cells with more than 95% of viability were immediately used for assays.

$CD4^+CD25^+CD127^{-/dim}$ (Treg) and $CD4^+CD25^-$ (Teff) cell populations were purified as previously described [10]. Briefly, non-$CD4^+$ and $CD127^{high}$ cells were magnetically labelled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads and were subsequently depleted by negative selection. Pre-enriched $CD4^+$ T cells were then labelled with anti-CD25 microbeads. Subsequently, Treg cells were isolated by positive selection and $CD4^+CD25^-$ Teff cells that were not retained in the magnetic separation column during purification of $CD25^+$ cells