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# Innate and adaptive immune responses in chronic hepatitis C virus and hepatitis B virus infection with high viral loads

Yutaka Kishida
Division of Gastroenterology and Hepatology, Department of Internal Medicine, Osaka Kaisei Hospital, 1-6-10 Miyahara, Yodogawa-Ku, Osaka City, Osaka 532-0003 Japan.
Correspondence: E-mail: y-kishida@muse.ocn.ne.jp
Tel: +81-6-6393-6234

Abstract
Background: Cytokines and chemokines are critical regulators of innate and adaptive immunities during viral infection. We examined innate and adaptive immune responses to hepatitis C virus (HCV) and hepatitis B virus (HBV) at baseline and against controls. Methods: Twenty-seven cytokines were evaluated before treatment in 27 patients with chronic hepatitis C(CHC) [genotype1 (n=20), genotype2 (n=7), HCVRNA 5.72±3.17LogIU/ml] and 12 chronic hepatitis B(CHB) [e-antigen (Ag) (+) (n=5), e-Ag (-) (n=7), HBVDNA 6.19±1.31Logcopies/ml] and against controls(n=5). Results: Th1 and Th2 cytokines were significantly higher ($p<0.05$) in CHB than in CHC. The levels of IL-IL10 in CHC and CHB, and IL15 in CHC(genotype2) and CHB were significantly lower ($p<0.05$) than in controls. The levels of CXCL8 in CHC and CHB, IL12 in CHC and CHB [e-Ag (-)] and CXCL10 in CHC and CHB were significantly higher ($p<0.05$) than in controls. IFN-$\gamma$ was higher in CHB than in controls. Conclusion: Cytokines levels differed between CHB and CHC before treatment. Innate immune responses were impaired in CHB with HBeAg(-) and CHC, but not in CHB with HBeAg(+) with high viral loads. Adaptive immune responses were impaired in CHB and CHC and appear to reflect the distinct state of virus-host immune interactions between CHB and CHC.

Key words: chronic hepatitis C, chronic hepatitis B, innate immune response,

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adaptive immune response, cytokine, chemokine.

## 1. Introduction

Microbial infections are recognized by the innate immune system both to elicit immediate defense and to generate long-lasting adaptive immunity. The sensing pathways of the innate immune system induce the activation of relevant effector responses of the adaptive immune system [1]. Host immune responses need to be tightly regulated by an intricate balance between positive and negative signals while combatting against pathogens; persistent pathogens may usurp these regulatory mechanisms to dampen host immunity to facilitate survival *in vivo* [2]. The pathogenesis of hepatitis C virus (HCV) infection is strongly influenced by the nature of host antiviral immunity. Host immune responses play a key role in defining the clinical outcome of HCV infection [3].

Cytokines are intercellular mediators involved in viral control and liver damage being induced by infection with HCV infection. The complex cytokine network operating during the initial stage of infection allows for the co-ordinated and effective development of innate and adaptive immune responses. Cytokines play an important role in the pathogenesis of cirrhosis and hepatocellular carcinoma (HCC), most cases of which are related to either hepatitis B virus (HBV) or HCV. Patients with HBV or HCV infection, with or without HCC, have distinctly different cytokine profiles, suggesting potential differences in disease pathogenesis and/or disease characteristics [4]. HCV interferes with cytokines at various levels and escapes immune responses by inducing a Th2/T cytotoxic 2 cytokines [5, 6].

However, the precise mechanisms responsible for the high rates of viral persistence have not yet been elucidated in detail [3]. The expression profiles of serum cytokines and chemokines, which are associated with the outcomes of patients in response to anti-HCV treatment currently remain unclear. Cytokines activating NK cells responses and their consorted effects in providing unique endogenous milieus promote downstream adaptive responses, which are the most beneficial in defenses against viral infections

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[6].

Persistent infection with HCV and HBV may lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [7]. Host immune responses to HBV and HCV are a critical factor affecting the outcome of HBV and HCV infection. Effective innate and adaptive immune responses are essential for the control of HCV infection [8]. Innate immune responses represent the front line of defense in humans. Primary defects in the innate immunity may shift the balance of immune responses towards dysregulated adaptive immunity, while the insufficient or excessive activation of innate immune response results in disease [9].

Factors leading to failed immune responses to clear virus-infected hepatocytes have not yet been elucidated in detail. A deeper understanding of the mechanisms responsible for the local regulation of antiviral immunity in the liver that affects clearance or persistence is important for elucidating the pathogenesis of disease and developing future successful immune therapies [1]. The multiple mechanisms involved in the induction of immune responses are variations of a common design principle in which cells that detect infections produce one set of cytokines to induce lymphocytes in order to produce another set of cytokines, which in turn activate effector responses [10].

Cytokines are involved in communication between cells of the immune system and contribute to the activation of correct cell types that are appropriate for defense response to different classes of infective agent. Cytokines are also capable of inducing the maturation and differentiation of immune cell subsets into more specialized effector cell classes with unique capabilities to protect against particular types of infection [8].

In contrast to HBV, HCV may be completely cleared by immune responses. Key mediators of spontaneous HCV clearance are virus-specific T cells, which remains readily detectable in the circulation for decades after clearance. Innate immune response cells such as natural killer T (NKT) cells and natural killer (NK) cells comprise a major cell population in the liver, and have the capacity to respond rapidly to chemokines and/or to altered cell

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surface marker expression on infected cells. They may influence direct antiviral effector functions and assist in priming and modulating adaptive immune responses. NK cell activation may be mediated by inflammatory cytokines such as type I interferon (IFN)s and interleukin (IL)-12 which are commonly released in response to viral infections [11]. Viruses have developed several strategies to escape these immune responses. Escape from adaptive immune responses may be achieved by the emergence of viral escape mutations that avoid recognition by antibodies and T cells [12].

Chemokines and inflammatory cytokines are essential regulators of innate and adaptive immunities as well as inflammation during viral infections. Host cytokines and the innate immune responses play an important role in the regulation of HCV and HBV [13]. HCV impairs the activation of innate immune responses. A sustained HCV infection results from ineffective innate and adaptive immunities. Innate immune responses regulate adaptive immune responses through direct interactions and by means of the exchange of signals between immune cells [5, 11, 14, 15, 16]. However, the expression profiles of serum cytokines and chemokines have not been fully elucidated in chronic hepatitis B (CHB) and chronic hepatitis (CHC) patients relative to healthy controls. A better understanding of these profiles is crucial for broadening our knowledge on immune response to the pathogenesis of HCV and developing novel and effective immunotherapies for HCV. HCV infection has been associated with a cytokine imbalance [17].

Th1 and Th2 cytokines play a prominent role in viral infections and the dysregulation of these cytokines may account for viral persistence and the evolution of chronic disease [18]. HBV is transcriptionally silent in acute infections only the weak to no induction of host innate immune responses which is in contrast to HCV. HBV is a “stealth virus” that bypasses the innate sensing machinery during early infection. Furthermore, HBV is readily suppressed by innate immune components including IFN- $\alpha$ , toll-like receptors, NK cells, NKT cells, and antigen-presenting cells (APCs). The actual elimination of HBV infection requires adaptive immune responses.

Immune responses are considered to play a key role in the outcome of HBV

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infection. Although the mechanisms for the pathogenesis of liver disease have not yet been elucidated in detail, the levels of hepatitis and viral replication observed in various phases of CHB may reflect distinct states of virus-host immune interactions. Vertically HBV transmission from mothers to neonate is associated with an immune tolerance phase with HBe-antigen (Ag) status and high levels of viral replication without any liver enzyme elevation. After 1 to 2 decades, this phase may change to an HBeAg-positive (+) immune active phase with increased alanine aminotransferase levels and a fluctuating viral load that may convert into an HBeAg-negative (-) inactive carrier phase characterized by low levels of viral replication and low alanine aminotransferase as well as necro-inflammatory liver disease [19].

In the present study, we investigated the innate and adaptive immune responses in HBV and HCV infections at baseline before treatment and against controls. The present study aimed to clarify the expression patterns of cytokines and chemokines in chronic HCV and HBV infections and against controls. Twenty-seven cytokines and chemokines were analyzed. Cytokines levels differed between CHB and CHC at baseline before treatment. Innate immune responses were impaired in CHB with HBeAg (-) and CHC, but not in CHB with HBeAg (+) with high viral loads. Innate and adaptive immune responses were stronger in CHC (genotype 2) than in CHC (genotype1). Adaptive immune responses were impaired in CHB and CHC and appear to reflect the distinct state of virus-host immune interactions among CHB with HBeAg (+), CHB with HBeAg (-), CHC (genotype 1) and CHC (genotype 2). Patients with CHB exhibited a distinct immune-regulatory cytokine pattern that shifted towards the Th1 arm [20]. Skewed cytokines and chemokines expression at baseline before treatment was observed in CHB with HBeAg (+), CHB with HBeAg (-), CHC (genotype 1) and CHC (genotype 2), and may play an important role in persistent HCV and HBV infections. A better understanding of the pathogenesis of chronic HCV and HBV infection may be obtained by exploiting the expression pattern of cytokines and chemokines [21].

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## 2. Methods

### 2-1. Study Subjects

We performed a prospective study on 27 patients with CHC with fibrosis stage of F1~2 [12 men and 15 women, aged 57.74 ±12.00 yr., genotype 1 (n=20) and genotype 2 (n=7), HCV RNA 5.72 ± 3.17 LogIU/ml, Platelet counts 17.1 ± 10.0 x10$_4$/ $\mu$ l, Fib 4 indexes 3.20 ± 2.35 and APRI scores 1.27 ± 1.35] and 12 patients with CHB with fibrosis stage of F1~2 [7 men and 5 women, aged 47.5 ± 13.50 yr., HBeAg (+) (n=5) and HBeAg (-) (n=7), genotype B (n=2), genotype C (n=9) and the genotype of one patient was not elucidated, HBV DNA 6.19 ± 1.31 Log copies/ml, Platelet counts 18.6 ±6.6 x10$_4$/ $\mu$ l, Fib 4 indexes 2.17 ± 1.78 and APRI scores 0.76 ± 0.48] who were enrolled before treatment between October 2004 and February 2010. They had not received any antiviral treatment in the preceding 12 months. Patients characteristics are shown in Tables 1 and 2. Serum samples were collected obtained from 27 patients with CHC, 12 patients with CHB and 5 healthy controls, and stored at – 20 degrees Celsius.

Written informed consent was obtained from all patients according to the Declaration of Helsinki.

### 2-2. Measurements

Routine biochemical and hematological tests were performed. HCV RNA was measured at baseline before treatment using the quantitative COBAS AMPLICORE HCV MONITOR test, ver. 2.0 (Roche Diagnostic System, Tokyo, Japan, sensitivity < 50 IU/ml). HCV sero-groups were assessed by the HCV sero-grouping assay (HCV Gr; Sysmex International Reagents, Kobe, Japan), which can subgroup the patients in HCV sero-groups 1 and 2, corresponding to HCV genotypes 1 and 2, respectively, with HCV group-specific anti-nonstructural region 4 antibodies. This assay is available not only for patients with chronic HCV infection, but also for those with resolved HCV. HBV DNA was measured using the quantitative COBAS TaqMan PCR HBV-auto ver. 2.0 (Roche Diagnostic System, Tokyo, Japan, sensitivity < 2.1 Log copies /ml). HBV genotypes were

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elucidated in serum by the the restriction fragment length polymorphism (RFLP) method on the S-gene sequence amplified by a polymerase chain reaction (PCR) with nested primers.

2-3. Bio-Plex Multiplex cytokine assay

The serum cytokines and chemokines (multiple cytokine assay) were measured at baseline before treatment. A multiplex Enzyme-linked Immuno-sorbent Assay (ELISA)-based immunoassay, with dyed microspheres conjugated to a monoclonal antibody specific for a target PLex Human Cytokine assay (BioRad Inc., Tokyo Japan) was used. A broad range, 1.95-32,000 pg/ml, of standards was used to establish standard curves to maximize the sensitivity and dynamic range of the assay. Cytokine levels were determined using a Bio-Plex array reader (an automated flow-based microfluidics device that uses a dual-laser fluorescent detector with real-time digital signal processing for quantitation; Luminex, Austin, TX, U.S.A.). This instrument quantitates multiplex immunoassays in a 96-well format using very small serum volumes (12.5 $\mu$ l). The concentrations of analyses in these assays were calculated using a standard curve with software provided by the manufacturer. Regression analysis was performed to derive an equation that was then used to predict the concentration of cytokines in serum samples. The cytokines measured were Th1 cytokines [IFN- $\gamma$ , TNF- $\alpha$ , IL1- $\alpha$ , IL-1 $\beta$ , IL-2, IL-7, IL-12(p70), IL-15 and IL-17], Th2 cytokines [IL-4, IL-6, IL-9, IL-10, and IL-13], CXC chemokines [CXCL-8 (IL-8) and CXCL-10 (IP-10)], CC chemokines [CCL-2 (MCP-1), CCL-3 (MIP-1 $\alpha$ ), CCL-4 (MIP-1 $\beta$ ), CCL-5 (RANTES) and CCL-11 (Eotaxin)], G-CSF, VEGF and PDGF.

2-4. Statistical Analysis

Data were expressed as the mean ± standard deviation. Relationships between variables were tested using the Student's *t*-test to evaluate differences, with a $p$ value of $< 0.05$ being considered significant.

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# 3. Results

## 3-1. Study subjects at baseline before treatment

Subject characteristics are shown in Tables 1 and 2.

**TABLE 1. Patients Characteristics of Chronic Hepatitis C**

| Characteristics | All patients | genotype 1 | genotype 2 | |
|---|---|---|---|---|
| Baseline | (n=27) | (n=20) | (n=7) | *P* |
| Age, years | 57.74±12.00 | 57.20 ±12.10 | 59.30±12.40 | 0.701 |
| Male, n (%) | 12 (44.4%) | 8 (40.0%) | 4 (57.1%) | 0.432 |
| BMI , kg/m$^2$ | 22.56 ±3.59 | 23.0 ±3.40 | 21.42 ±4.0 | 0.303 |
| Treatment history | | | | |
| previous IFN | 8 (29.6%) | 7 (35.9%) | 1 (14.3%) | |
| Laboratory results | | | | |
| HCV.RNA (LogIU/ml) | 6.26 ± 0.80 | 6.10 ± 1.50 | 6.20± 0.70 | 0.857 |
| AST (IU/ml) | 67.6 ± 48.8 | 58.5 ± 43.2 | 73.4± 35.3 | 0.419 |
| ALT (IU/ml) | 79.1 ± 60.9 | 62.5± 53.4 | 89.4 ± 49.7 | 0.254 |
| Platelets ($10^4$ / $\mu$l) | 17.1 ± 10.0 | 18.2± 9.1 | 16.7 ± 3.7 | 0.654 |
| Fib 4 index | 3.20 ± 2.35 | 3.28 ± 2.62 | 3.01 ±1.50 | 0.804 |
| APRI score | 1.27 ± 1.35 | 1.20 ± 1.54 | 1.24 ± 0.76 | 0.949 |

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**TABLE 2. Patients Characteristics of Chronic Hepatitis B**

| Characteristics | All patients | HBe-antigen positive | HBe-antigen negative | |
|---|---|---|---|---|
| Baseline | (n=12) | (n=5) | (n=7) | *P* |
| Age, years | 47.5±13.5 | 54.8 ±15.1 | 42.3 ±10.4 | 0.117 |
| Male, n (%) | 7 (58.3%) | 1 (20.0%) | 6 (85.7%) | 0.023 |
| BMI , kg/m$^2$ | 22.56 ±3.59 | 21.7 ±2.7 | 26.8 ±5.6 | 0.091 |
| HBV-genotype | | | | |
| genotype B | 2 (16.7%) | 1 (20.0%) | 1 (14.3% ) | |
| genotype C | 9 (75%) | 4 (80.0%) | 5 (71.4%) | |
| not determined | 1 (8.3%) | 0 (0.0%) | 1 (14.3%) | |
| Laboratory results | | | | |
| HBV.DNA (Log copies/ml) | 6.19 ± 1.31 | 6.80 ± 0.50 | 5.80 ± 1.60 | 0.181 |
| AST (IU/ml) | 46.7 ± 20.2 | 60.8 ± 10.2 | 36.6 ± 19.8 | 0.032 |
| ALT (IU/ml) | 55.6 ± 30.0 | 66.4 ± 26.1 | 47.9 ± 32.0 | 0.313 |
| Platelet ($10^4$ / $\mu$ l) | 18.6 ± 6.6 | 14.2 ± 6.1 | 21.7 ± 5.4 | 0.048 |
| Fib 4 index | 2.17 ± 1.78 | 3.65 ± 1.88 | 1.11 ±0.58 | 0.007 |
| APRI score | 0.76 ± 0.48 | 1.18 ± 0.34 | 0.471 ± 0.343 | 0.005 |

3-2. Serum cytokines and chemokines at baseline before treatment:

Serum levels of IL-1 $\alpha$ in CHB (all patients with CHB) were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of IL-1 $\alpha$ were higher in CHB with HBeAg(-) than in CHB with HBeAg(+) (Figure 1A). Serum levels of Th1 cytokines (IL-2, IL-12 and IL-15) were significantly higher ($p < 0.05$) in CHB (all patients with CHB) than in CHC (all patients with CHC) (Figure 1B, 1C, 1D). Serum levels of IL2 were higher in CHB with HBeAg(+)than in CHB with HBeAg(-) (Figure 1B). Serum levels of IL-12 were higher in CHC (genotype 2) than in CHC (genotype 1). Serum levels of IL-12 were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 1C). Serum levels of IL-15 were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 1D).

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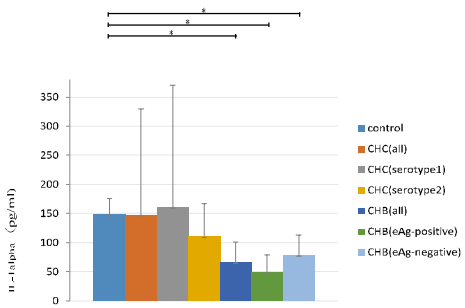
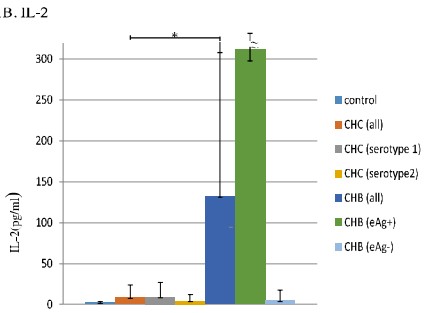
1A. IL-1α




1B. IL-2



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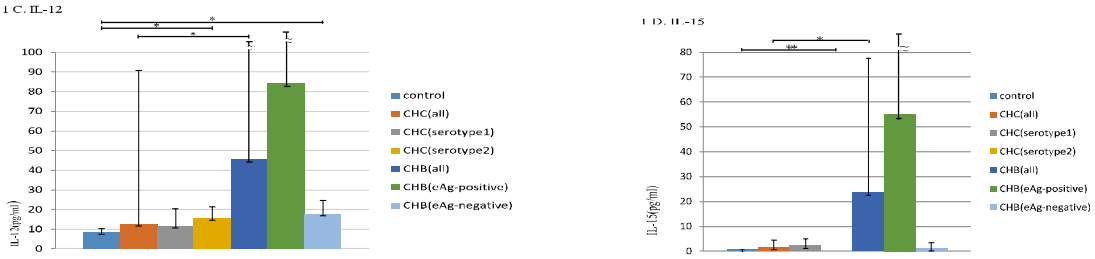


Figure 1. Histograms represent serum Th1 cytokines [Figure 1A (IL- $\alpha$ 1), Figure 1B (IL-2), Figure 1C (IL-12), Figure 1D (IL15) ] expression in patients with HCV serotype 1 (genotype1) and serotype 2 (genotype2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$. ** $p < 0.01$

Serum levels of IL-4 were higher in CHB with HBeAg(+) than CHB with HBeAg(-) (Figure 2A). Serum levels of IL-6 were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 2B). Serum levels of IL-9 were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 2C). Th2 cytokines (IL-4, IL-6 and IL-9) (Figure 2A, 2B, 2C) were significantly higher ($p < 0.05$) in CHB (all patients with CHB) than in CHC (all patients with CHC).

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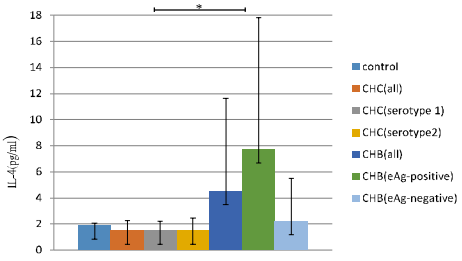
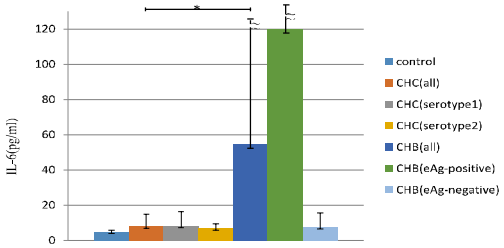
2 A. IL-4




2B. IL-6



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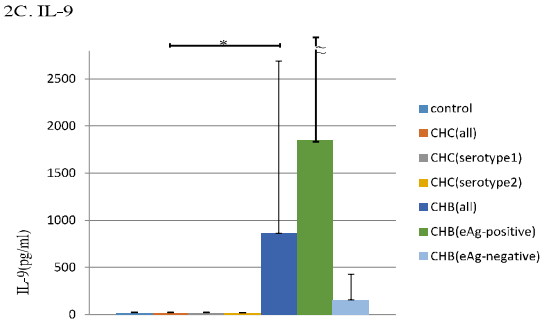


Figure 2. Histograms represent serum Th2 cytokines [Figure 2A (IL-4), Figure 2B (IL-6), Figure 2C (IL-9)] expression in patients with HCV serotype 1 (genotype1) and serotype 2 (genotype2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment.

* $P < 0.05$. ** $p < 0.01$

Serum levels of CCL-3 (MIP1-$\alpha$) were significantly higher ($p < 0.05$) in CHB (all patients with CHB) than in CHC (all patients with CHC). Serum levels of CCL-3 (MIP1-$\alpha$) were higher in CHB with HBeAg (+) than in CHB with HBeAg (-) (Figure 3).

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Serum CCL-3 (MIP-1alpha) expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

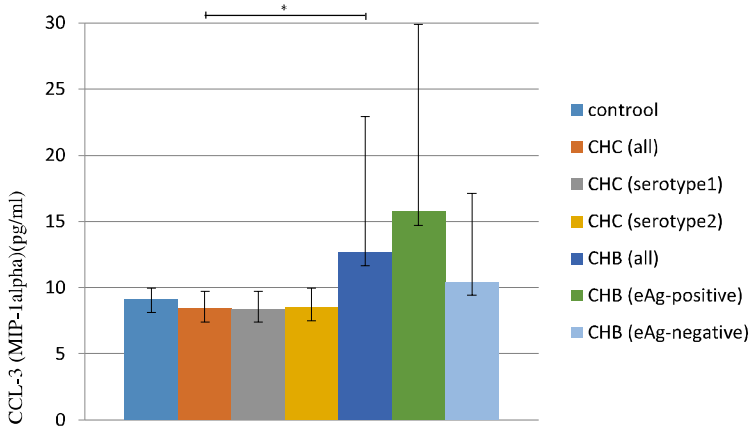


Figure3. Histograms represent serum cytokine [CCL-3 (MIP-1alpha)] expression in patients with HCV serotype 1 (genotype1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$

Serum levels of IL1 $\beta$ in CHC (genotype 2) were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of IL-1 $\beta$ were higher in CHC (genotype 1) than in CHC (genotype2). Serum levels of IL-1 $\beta$ were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 4).

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Serum IL-1 $\beta$ expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

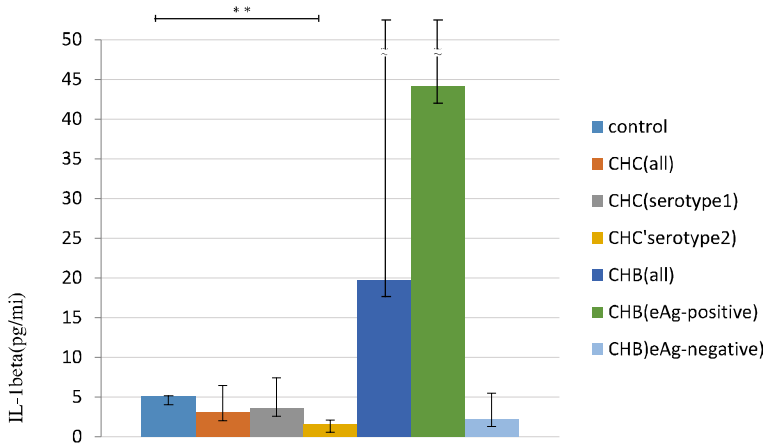


Figure 4. Histograms represent serum cytokine (IL-1 $\beta$ ) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. ** $P < 0.01$.

Serum levels of IL-7 in CHC (all patients with CHC) were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of IL-7 were higher in CHB with HBeAg(+) than in CHB with eAg(-). Serum levels of IL-7 were higher in CHC (genotype 1) than in CHC (genotype2) (Figure 5).

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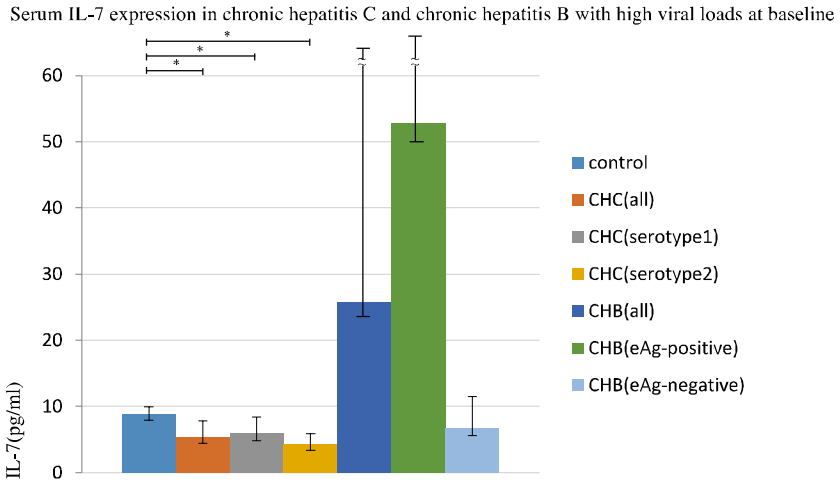


Figure 5. Histograms represent serum cytokine (IL-7) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$.

Serum levels of IL-10 in CHC (all patients with CHC, genotype 1 and genotype 2) were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of IL-10 were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 6).

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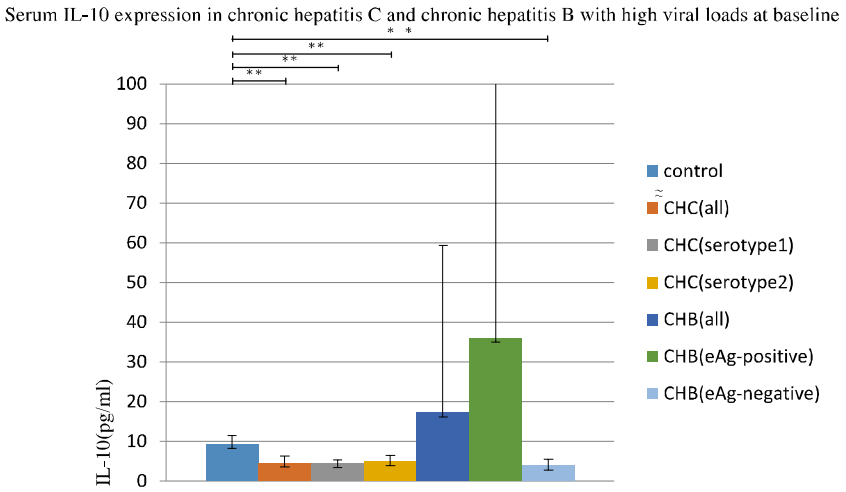


Figure 6. Histograms represent serum cytokine (IL-10) expression in patients with HCV serotype 1(genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. ** $P < 0.01$

Serum levels of IL-13 in CHC (all patients with CHC, genotype 1 and genotype 2) and CHB with HBeAg(-) were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of IL-13 were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 7).

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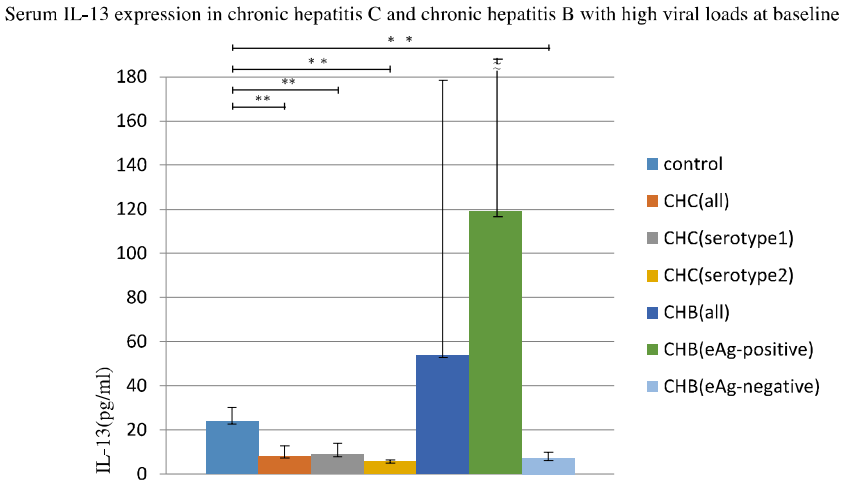


Figure 7. Histograms represent serum cytokine (IL-13) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S .D.). All the samples were tested for baseline levels before treatment. ** P < 0.01

Serum levels of G-CSF in CHC [all patients with CHC and CHC (genotype 2)] and CHB [all patients with CHB and CHB with HBeAg (-)] were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of GSF were higher in CHC (genotype 1) than in CHC (genotype 2). Serum levels of GSF were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 8).

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Serum GCSF expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

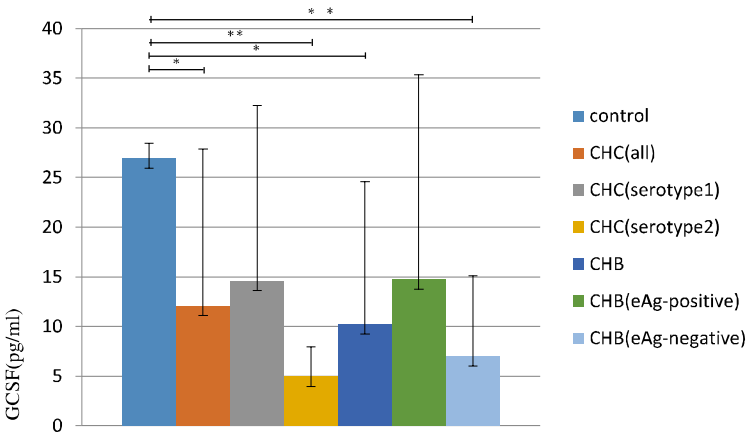


Figure 8. Histograms represent serum cytokine (GCSF) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$ * * $P < 0.01$

Serum levels of IFN- $\gamma$ in CHC (genotype 2) were significantly lower ($p < 0.05$) than those in healthy controls. Serum levels of IFN- $\gamma$ were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 9).

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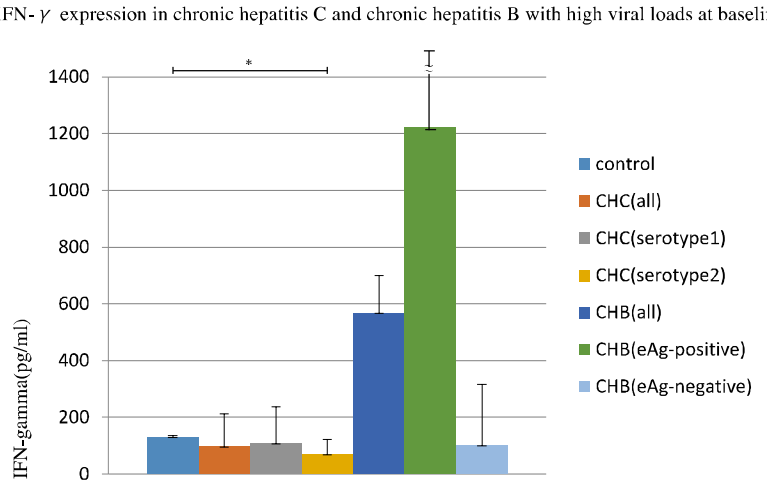


Figure 9. Histograms represent serum cytokine (IFN-γ) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$

Serum levels of VEGF in CHC (all patients with CHC, genotype 1 and genotype 2) and CHB [all patients with CHB, and CHB with HBeAg(+), and CHB with HBeAg(-)] were significantly lower ($p < 0.05$) than those in healthy controls (Figure 10).

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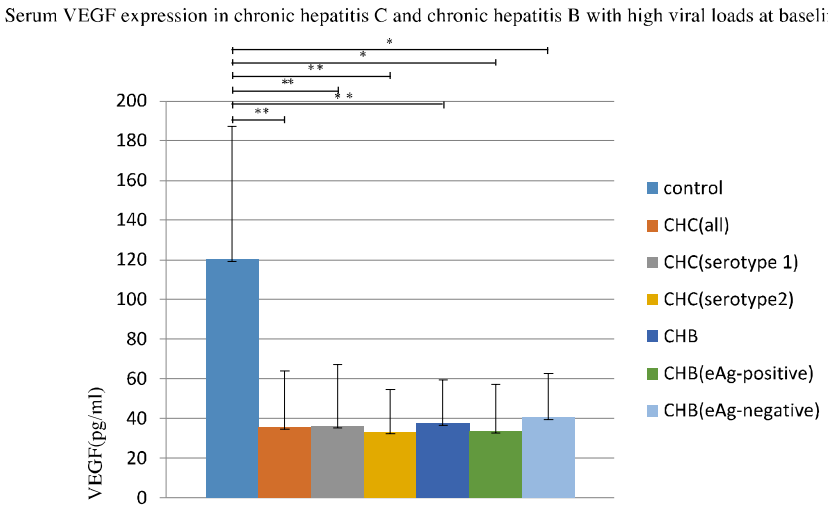


Figure10. Histograms represent serum cytokine (VEGF) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment . *P < 0.05, ** P < 0.01

Serum levels of CXCL8 (IL-8) in CHC [all patients with CHC, CHC (genotype 1) and CHC (genotype 2)] and CHB [all patients with CHB, CHB with HBeAg (-) and CHB with HBeAg(-)] were significantly higher ($p < 0.05$) than those in healthy controls. Serum levels of CXCL8 (IL-8) were higher in CHC (genotype 2) than in CHC (genotype 1) (Figure 11).

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Serum CXCL-8(IL-8) expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

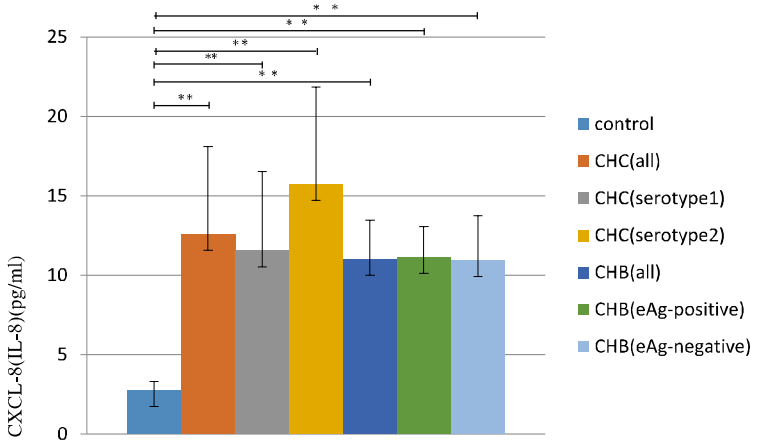


Figure 11. Histograms represent serum cytokine [CXCL-8(IL-8)] expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. ** P < 0.01

Serum levels of IL-17 in CHB with HBeAg (+) were significantly lower ($p<0.05$) than those in healthy controls. Serum levels of IL-17 were significantly higher ($p < 0.05$) in CHC (all patients with CHC) than those in CHB (all patients with CHB). Serum levels of IL-17 were higher in CHC (genotype 2) than in CHC (genotype 1) (Figure 12).

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Serum IL-17 expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

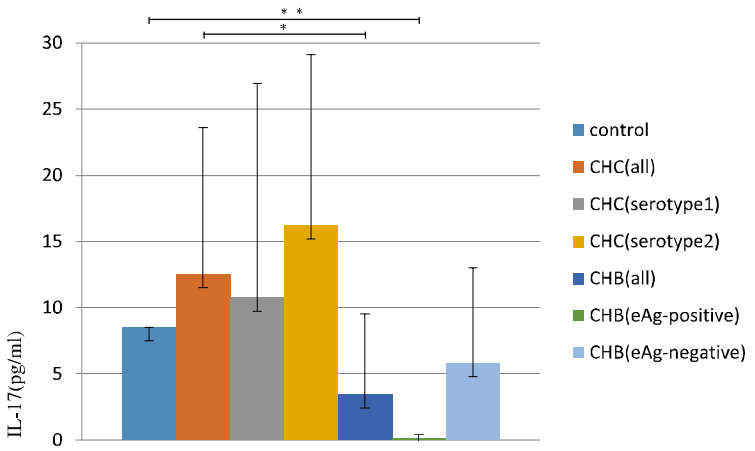


Figure12. Histograms represent serum cytokine (IL-17) expression in patients with HCV serotype 1(genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. *P < 0.05, ** p < 0.01

Serum levels of CCL-2 (MCP1) in CHC [all patients with CHC, CHC (genotype 1) and CHC (genotype 2)] and CHB [HBeAg (-)] were significantly higher ($p < 0.05$) than those in healthy controls. Serum levels of CCL-2 (MCP1) were higher in CHC (genotype 2) than in CHC (genotype 1). Serum levels of CCL-2 (MCP1) were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 13).

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Serum CCL-2 (MCP-1) expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

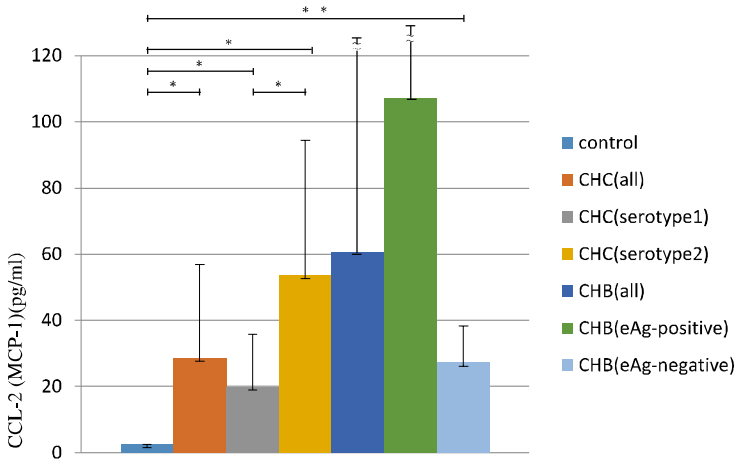


Figure13. Histograms represent serum cytokine [CCL-2 (MCP-1)] expression in patients with HCV serotype 1(genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. *P < 0.05, ** p < 0.01

Serum levels of CXCL-10 (IP-10) were significantly higher ($p < 0.05$) in CHB [all patients with CHB, CHB with HBeAg (+) and CHB with HBeAg (-)] than those in healthy controls. Serum levels of CXCL-10 (IP-10) were significantly higher ($p < 0.05$) in CHC (all patients with CHC) than in CHB (all patients with CHB). Serum levels of CXCL-10 (IP-10) were higher in CHC (genotype 1) than in CHC (genotype 2). Serum levels of CXCL-10 (IP-10) were higher in CHB with HBeAg(+) than in CHB with HBeAg(-) (Figure 14).

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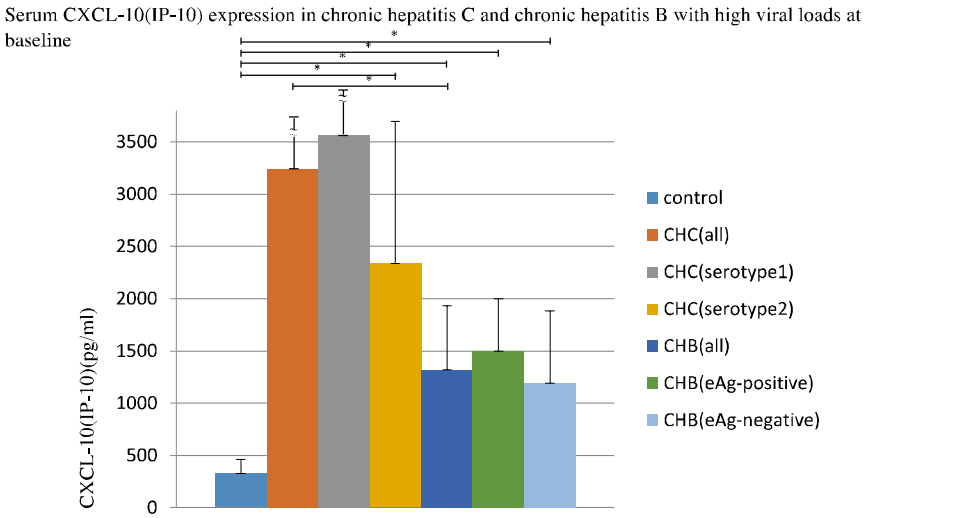


Figure 14. Histograms represent serum cytokine [CXCL-10(IP-10)] expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$

Serum levels of CCL-4 (MIP1 $\beta$ ) in CHC [all patients with CHC, CHC (genotype 1) and CHC (CHC (genotype 2) ) and CHB [all patients with CHB and CHB with HBeAg (+) ]were significantly higher ($p < 0.05$) than those in healthy controls. Serum levels of CCL-4 (MIP1 $\beta$ ) were significantly higher ($p < 0.05$) in CHC (genotype 2) than in CHC (genotype 1). Serum levels of CCL-4 (MIP1 $\beta$ ) were higher in CHB with HBeAg(-) than in CHB with HBeAg(+) (Figure 15).

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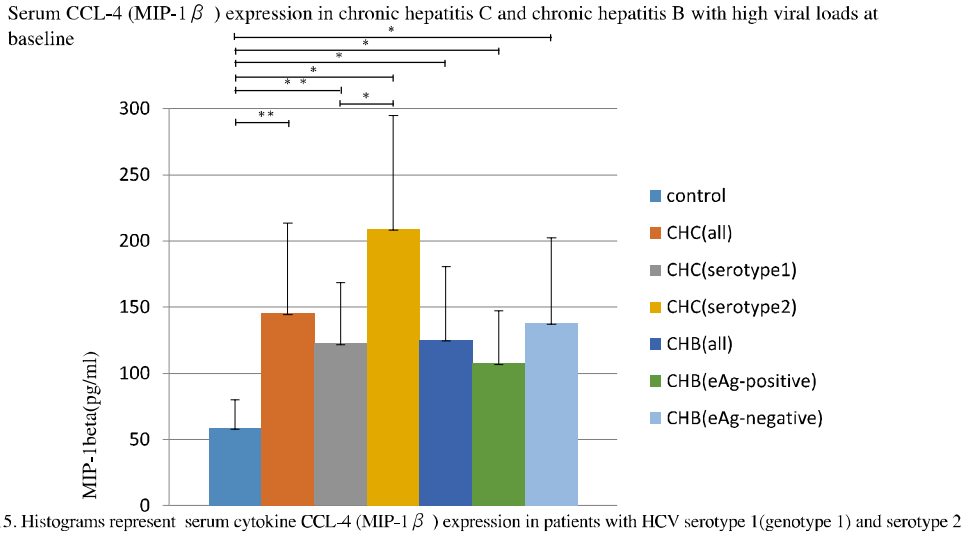


Figure15. Histograms represent serum cytokine CCL-4 (MIP-1β ) expression in patients with HCV serotype 1(genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. *P < 0.05, ** P < 0.01

Serum levels of CCL-5 (RANTES) in CHC [all patients with CHC and CHC (genotype 2)] and CHB [all patients with CHB and CHB with HBeAg(+) and CHB with HBeAg(-)] were significantly higher ($p < 0.05$) than those in healthy controls. Serum levels of CCL-5 (RANTES) were higher in CHC (genotype 2) than in CHC (genotype 1). Serum levels of CCL-5 (RANTES) were higher in CHB with HBeAg(-) than in CHB with HBeAg(+) (Figure 16).

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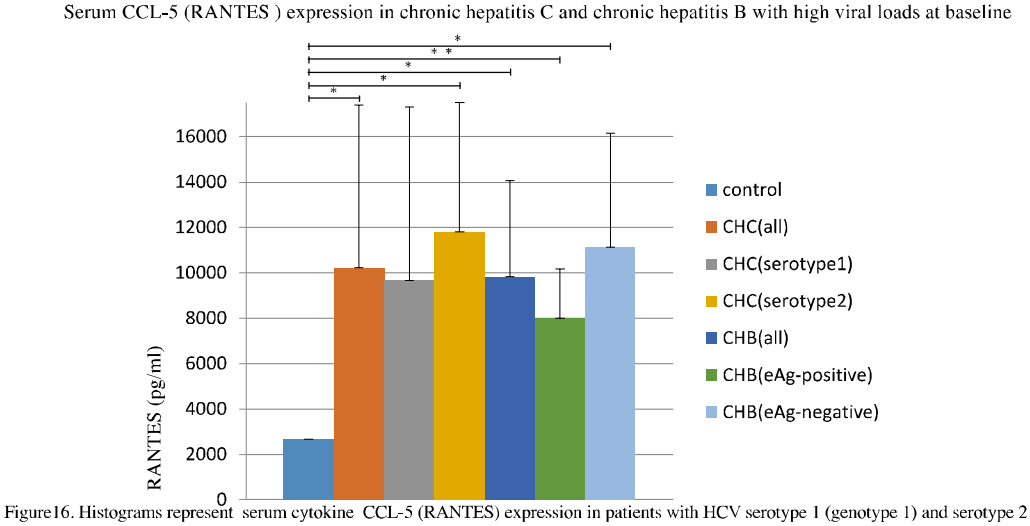


Figure16. Histograms represent serum cytokine CCL-5 (RANTES) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. *P < 0.05, ** P < 0.01

Serum levels of PDGF in CHB [HBeAg (-)] were significantly higher ($p < 0.05$) than those in healthy controls. PDGF in CHB [HBeAg (-)] was significantly higher ($p < 0.05$) than those in CHB [HBeAg (+)] (Figure 17).

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Serum PDGF expression in chronic hepatitis C and chronic hepatitis B with high viral loads at baseline

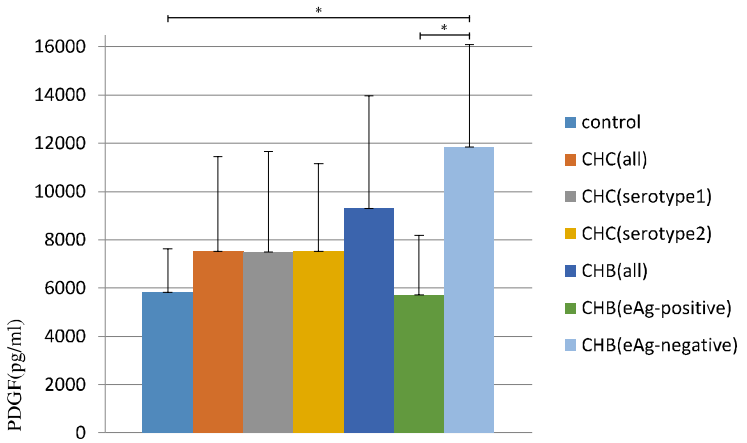


Figure17. Histograms represent serum cytokine (PDGF) expression in patients with HCV serotype 1 (genotype 1) and serotype 2 (genotype 2) infection with high viral loads, and HBV e-antigen positive and e-antigen negative infection with high viral loads, and healthy controls (mean ± S.D.). All the samples were tested for baseline levels before treatment. * $P < 0.05$

## 4. Discussion

The present study aimed to clarify the expression patterns of cytokines and chemokines in CHC and CHB at baseline before treatment and against controls. Twenty-seven patients with CHC and 12 with CHB were enrolled in this study. Serum levels of Th1/Th2 cytokines and chemokines were measured.

IL-12 levels were significantly higher ($p < 0.05$) in CHB than in CHC as well as in CHC (genotype 2) and CHB with HBeAg (-) than in controls and were higher in CHC (genotype 2) than in CHC (genotype 1) (Figure 1C). CHB with HBeAg (+) had higher serum levels of IL-12 than CHC and controls, suggesting that IL-12 production is not impaired in CHB. The present results demonstrated that innate immune responses were stronger in CHB with HBeAg (+) than in CHB with HBeAg (-). Furthermore, innate and adaptive immune responses were stronger in CHC (genotype 2) than in CHC (genotype 1). IL-12 may play an important role in inducing Th1 cytokine responses and HBeAg seroconversion, and viral clearance in chronic HBV infection. IL-12 has been suggested to contribute to viral clearance in CHB

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[22]. Cellular immune responses are deficient in HCV-infected patients and IL-12 may enhance these responses [23]. A switch to the Th1-type of responses would be beneficial and promote essential cellular immune mechanisms for viral clearance. In transgenic mice expressing HBeAg, the systemic application of recombinant IL-12 resulted in a shift from Th2-mediated responses towards a predominance of the Th1 cytokine profile. Although IL-12 production is not impaired in CHB, marked increase in IL-12 production was shown to be necessary for the induction of Th1 cytokine responses and ultimately HBeAg sero-conversion [22]. Host immune responses may affect the outcome of HCV infection and IL-12 plays an essential role in host defenses against infectious diseases.

Serum levels of IL-12, IL-15, and IL-17 were elevated in CHC than in controls. IFN-$\gamma$ levels were found to be lower in CHC than in CHB and controls (Fig 9). In addition to inducing NK cell IFN-$\gamma$ production, the innate cytokine IL-12 may contribute to the preferential development of Th1 cells that produce IFN-$\gamma$ over Th2 cells producing IL-4 and IL-5 [24]. NK cells constitute the main innate immune cell population in the liver [25]. These results demonstrated that innate immune responses were weaker in CHC than in CHB. NK cell IFN-$\gamma$ is an innate cytokine. NK cell cytotoxicity during infections is dependent upon the IL-12 to NK cells IFN-$\gamma$ pathway.

IL-15 levels were significantly higher ($p < 0.05$) in CHB with HBeAg (+) than in CHC(all patients with CHC) (Fig1D). IL-15 functions as a differentiation factor for NK-cells [3], is an important cytokine in innate and adaptive immune responses against HCV infection [26], and may function as an NK cell growth and maturation factor to drive the proliferation of NK cells and memory T cells [6].

IL-10 levels were higher in CHB with HBeAg (+) than in CHB with HBeAg (-), CHC [all patients with CHC, CHC (genotype 1) and CHC (genotype2)] and controls. IL-10 levels were significantly lower ($p < 0.05$) in CHC and CHB with HBeAg (-) than in controls (Figure 6). IL-10 and TGF-$\beta$ are involved in the negative regulations of NK cells during innate immune

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responses. IL-10 is induced under certain conditions of innate immunity to negatively regulate other pro-inflammatory cytokines. IL-4, IL-6 and IL-9 levels were significantly lower ($p < 0.05$) in CHC than in CHB and controls (Figure 2A, 2B, 2C). IL-4 and IL-6 are critical and for the production of the Th2 cell phenotype [20], IL-9 enhances the growth of monocytes, synergizes with IL-4 in the switch to IgG1 [27].

IL-17 levels were significantly higher ($p < 0.05$) in CHC than in CHB. Serum levels of IL-17 were higher in CHC (genotype 2) than in CHC (genotype 1) (Figure 12). IL17 acts as a link between innate and adaptive immune responses [28].

HCV encodes several strategies to evade antiviral responses, and this evasion of innate immunity plays a key role in determining viral persistence [29].

Serum levels of CXCL-8 (IL-8) levels were significantly higher ($p < 0.01$) in CHC and CHB than in controls. Serum levels of CXCL-8 (IL-8) were higher in CHC (genotype 2) than in CHC (genotype 1) (Figure 11). HCV infection induces CXCL-8 (IL-8), which is regulated at the levels of transcription and mRNA stability [30]. CCL-2 (MCP-1) levels were significantly higher ($p < 0.05$) in CHC and CHB than in controls. CCL-2 (MCP-1) levels were higher in CHC (genotype 2) than in CHC (genotype 1). CCL-2 (MCP-1) levels were higher in CHB with HBeAg(+) than in CHB with HBeAg (-) (Figure 13). CCL-2 (MCP-1) induces monocytes to leave the bloodstream and enter the surrounding tissues to become tissue macrophages. Serum levels of CCL-5 (RANTES) levels were significantly higher ($P < 0.05$) in CHC and CHB than in controls. Serum levels of CCL-5 (RANTES) levels were higher in CHC (genotype2) than in CHC (genotype 1) (Figure 16). CCL-5 (RANTES) attracts cells such as T cells, eosinophils and basophils that express the receptor CCR5 [31]. CCL-4 (MIP-1 $\beta$ ), CCL-1 (MCP-1) and CCL-5 (RANTES) levels were significantly higher ($p < 0.05$) in CHC and CHB than in controls. Serum levels of CCL-4 (MIP-1 $\beta$ ) and CCL-1 (MCP-1) were significantly higher ($p < 0.05$) in CHC (genotype 2) than in CHC(genotype1). (Figures 13, 15, 16). The present results showed

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that innate immune responses were impaired in CHB with HBeAg (-) and CHC, but not impaired in CHB with HBeAg (+); however, adaptive immune responses were impaired in CHB with HBeAg (+) and CHC. Serum levels of VEGF and G-CSF were significantly lower ($p < 0.05$) in CHC and CHB than in controls. Serum levels of G-CSF were higher in CHC (genotype 1) than CHC(genotype2) (Figures 13, 15). VEGF and G-CSF are modulators of innate immune responses with suppressive effects [32, 33]. Serum levels of PDGF were higher in CHC and CHB than in controls (Figure 17). PDGF stimulates chemotaxis and accelerates the formation of the extracellular matrix and collagen [34]. Chemokines are considered to be pro-inflammatory and may be induced during immune responses in order to recruit cells of the immune system to a site of infection. The major role of chemokines is as chemo-attractants that guide the migration of cells. They are released by many different cell types and guide cells of the innate and adaptive immune systems. CCL-3 (MIP-1 $\alpha$), CCL-4 (MIP-1 $\beta$), CCL-2 (MCP-1), CCL-5 (RANTES) and CXCL-10 (IP-10) levels were previously reported to be elevated in chronically infected mice, and suggested to participate in the continuous recruitment of monocytes, NK cells, and T cells to the liver. The role of NK cells in mediating viral clearance vs persistence remains unclear: they are essential for killing virally infected cells [35].

We previously confirmed the restoration of innate immune responses with a viral load decline, as indicated by the up-regulation of IL-12, and IL-15 and down-regulation of CXCL-8, CCL-4, and CXCL-10, however, improvement in adaptive immune responses were insufficient as indicated by the down-regulation of IFN $\gamma$ and TNF- $\alpha$ in CHC patients [36].

Host immune responses to HBV are a critical factor affecting the outcome of HBV infection. CHB show a defective early innate immune response, which are essential for the further induction of HBV-specific adaptive immunity by liver or immune cells and may contribute to the persistence of CHB or a weakened capacity to clear HBV [22]. Although HBV infects hepatocytes, the mechanisms responsible for immune responses against the virus and how it affects disease progression remain unclear. Viral loads were

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previously shown to affect the quality of the anti-HBV immune responses and outcomes of viral infections [35]. HBV replication is controlled by cytokines with IFNs, and IFN-$\gamma$ and TNF-$\alpha$ are key mediators [37]. NK cells constitute a major cellular arm of the innate immune system. IFN-$\gamma$ clears HBV-infected hepatocytes through non-cytolytic mechanisms. Therefore, NK cell-derived IFN-$\gamma$ may constitute an antiviral mechanism in the liver [38]. Immune responses are considered to play a key role in the outcome of HBV infection. Although the underlying pathogenic mechanisms for liver disease currently remain unclear, the levels of hepatitis and viral replication observed in various phases of CHB are considered to reflect distinct states of virus-host immune interactions [19]. Innate immune cells, such as neutrophils, macrophages, innate lymphoid cells, NK cells, and dendritic cells (DCs) are activated to produce cytokines and chemokines that recruit other immune cells which exert inflammatory and antiviral effects. Innate immune activation is also critical in the development of adaptive immune responses. HBV is transcriptionally silent in acute infection with the weak to no induction of host innate immune responses which is in contrast to HCV. HBV is a “stealth virus” that bypasses the innate sensing machinery during early infection. Furthermore, HBV is readily suppressed by innate immune components including IFN-$\alpha$, toll-like receptors, NK cells, NKT cells, and APCs. Innate immune cells are activated and contribute to the course of acute and chronic HBV infections. Early innate evasion by HBV may be important for the initial establishment of infection, rather than the persistence of HBV infection. The actual elimination of HBV infection requires the presence of adaptive immune responses [39]. In addition to initiating adaptive immune responses, innate immunity may be intricately involved in cooperation with adaptive immune functions. Thus, HBV may initially evade innate immune recognition to establish infection. However, multiple innate immune pathways are ultimately induced in CHB that influence clinical outcomes [40, 41, 42]. Changes in the balance of cytokine profiles may result in either persistence of HBV and HCV infections [43]. CHB with HBeAg (+) with high viral loads is more strongly associated with

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the activation of Th1-and Th2-type responses than CHB with HBeAg (-). Thus, preferential activation and commitment towards Th1- or Th2-cell subsets may influence the clinical consequences of HBV infection [22].

Immunity against HCV infection is known to be inadequate, because most cases transition to chronicity. HCV has evolved mechanisms to modulate and escape immune recognition by the host and the interplay between the innate and adaptive immune systems that affects the outcome of viral infection. In contrast to HBV, HCV may be completely cleared by an individual response. However, limited information is currently available on the mechanisms responsible for chemokine and pro-inflammatory cytokines responses to HCV and HBV infections. HBV is not directly cytotoxic to infected hepatocytes; the clinical outcome of infection results from complicated interactions between the virus and the host immune system [44]. Innate immunity plays an indispensable role in early virus infection, facilitating virus clearance. However, it has been reported that HBV is under-recognized and poorly eliminated by the innate immune system in the early stage of infection, possibly explaining the long-lasting persistence of viremia afterwards. The mechanism by which HBV evades innate immune recognition and establishes persistent infection remains a subject of debate. Besides, some researchers are becoming more interested in how to eradicate chronic HBV infection by restoring or boosting innate immunity [45].

A clearer understanding of these mechanisms is crucial to broadening our knowledge on immune responses to the pathogenesis of HCV and HBV infection and the development of novel and effective immunotherapies [43].

## 5. Conclusion:

HBV and HCV may both employ specific mechanisms to inhibit cytokine production, highlighting the critical roles of these molecules in recovery from viral infections. Cytokines levels differed between CHB and CHC at baseline before treatment. The baseline skewing of immune responses at baseline before treatment was observed in CHB and CHC with high viral loads as well as impairments to induce effective innate immune responses

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linked to adaptive immune responses. Innate immune responses were impaired in CHB with HBeAg (-) and CHC, but not CHB with HBeAg (+) with high viral loads. Innate and adaptive immune responses were stronger in CHC (genotype 2) than in CHC (genotype1). Adaptive immune responses were impaired in CHB and CHC and appear to reflect the distinct state of virus-host immune interactions among CHB with HBeAg(+), CHB with HBeAg(-), CHC (genotype 1) and CHC (genotype2). Therefore, different strategies need to be considered in the treatment and management of CHB and CHC with high viral loads. In CHC with high viral loads, the restoration of innate immune responses upon a viral load decline is crucial and the restoration of adaptive immune response also need to be considered, because of insufficient improvements in adaptive immune responses to regulate HCV infection. In addition to inducing a decline in HBV by restoring innate immune responses, the development of treatment that trigger adaptive immune response is needed to regulate HBV infection.

## Conflict of Interest

The author declares no conflict of interest.

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