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Expansion of HLA-G-Expressing Myeloid-Derived Suppressor Cells in Patients with Chronic Hepatitis B Virus Infection

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Abstract

Both human leukocyte antigen-G (HLA-G) and myeloid-derived suppressor cells (MDSCs) was associated with the pathogenesis of infectious diseases. Whether peripheral MDSCs express HLA-G during virus infection remains unknown. We investigated the frequency of HLA-G$^+$ MDSCs and its subsets in patients infected with chronic hepatitis B (CHB). In this study, frequencies of peripheral MDSCs (Lin1-HLA-DR-CD33$^+$CD11b$^+$) and HLA-G expressing subsets from 50 CHB patients and 27 normal controls were analyzed using flow cytometry. Data revealed the median percentage of MDSCs was not significantly different between the CHB patients and controls (0.30% *vs.* 0.29%; p=0.884). Among MDSCs, similar frequency was observed for CD14+ monocytic MDSC (mMDSCs; 31.25% *vs.* 23.35%; p=0.063) and CD15$^+$ granulocytic MDSC (gMDSCs; 22.60% *vs.* 21.55%; p=0.558) between the two groups. However, HLA-G$^+$ MDSCs was significantly increased in CHB patients compared with that of controls (3.30% *vs.* 0.50%; p<0.001). Furthermore, both HLA-G$^+$ mMDSCs (0.99% *vs.* 0.00%; p<0.001) and HLA-G$^+$ gMDSC (0.78% *vs.* 0.00%; p<0.001) were also dramatically increased in CHB patients. Particularly, HLA-G$^+$ gMDSC was inversely correlated to the viral DNA loads and significantly increased in HBeAb positive patients. Summary, this work reports for the first time HLA-G$^+$ MDSCs, a new population of peripheral MDSCs, were expanded in CHB patients; however, its clinical relevance yet to be further explored.

Keywords: HLA-G; Myeloid derived suppressor CELLS; HBV; Infection

Introduction

Hepatitis B virus (HBV) is one of the most common infectious agents for humans by affecting an estimated 240 million people worldwide [1]. HBV infection could cause liver damage such as acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma, which is particularly occurred in Asian countries [2]. During HBV infection, both innate and adaptive host immune response plays a critical role in pathogenesis and clinical outcomes of hepatitis B. Multiple immune cells, such as cytotoxic T lymphocytes, regulatory T cells, natural killer cells, dendritic cells and B cells are important in the immune regulation of HBV infection and determination whether HBV infection is cleared or persists [3,4].

Myeloid-derived suppressor cells (MDSCs), a heterogeneous population of granulocytic (gMDSC) or monocytic (mMDSC) cells, have been reported in a variety of virus infection associated pathologies [5]. The hallmark of MDSCs is their ability to suppress both innate and adaptive immune responses [6]. Suppressive factors expressed by MDSCs, such as arginase-1, reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and program death ligand 1(PDL-1), play pivotal roles in inhibition of T cell, B cell and natural killer cell (NK) mediated immune responses, and in induction of tolerogenic Tregs and regulatory dendritic cells (DCs) [7].

In the context of virus infection, hepatitis C virus (HCV) infection could promote peripheral MDSCs accumulation and suppress T cell responses [8,9]. Also, elevated peripheral MDSCs in chronic hepatitis C patients was observed to be related to HCV-RNA copies [10]. In Human immunodeficiency virus (HIV) infected patients, gMDSCs were expanded and could induce T-cell anergy by suppressing CD3ζ expression [11]. Garg et al. [12] found that CD4$^+$CD25$^+$FoxP3$^+$ regulatory T-cell could be induced by MDSCs promoted with the HIV type 1 gp120. In other pathogens, studies reported splenic MDSCs was increased with influenza A virus and vaccinia [13,14], and adenovirus can induce gMDSCs to suppress NK cell proliferation and activation via hydrogen peroxide in C57BL/6 mice [15]. These studies indicated that MDSCs could provide an additional means for virus to escape from host immune clearance.

The immunotolerant HLA-G is a member of the non-classical HLA class I antigen, and its ectopic expression was addressed in amounts of previous studies, where HLA-G was markedly increased during various virus infection and associated with disease progression [16]. HLA-G acts mainly through two inhibitory receptors including immunoglobulin-like transcript 2 (ILT2) and ILT4 [17]. The murine receptor paired immunoglobulin like receptor-B (PIR-B), a homology to human ILTs, can bind to HLA-G [18]. Previous findings had recognized that through binding to the PIR-B expressed on MDSCs, and HLA-G has been found with the capability to contribute the expansion and suppressive functions of MDSCs [19,20].

In addition to HLA-G exerts its regulatory functions directly by interacting with specific inhibitory receptors expressed on various immune cells, many kinds of immune cells can express HLA-G themselves which could be affected by micro environmental factors,

maturation stimuli and even by the process of trogocytosis, such as HLA-G-expressing APC, or MSC, or T or NK cells [21-24]. DC-10, a new subset of human tolerogenic DCs, is characterized by the expression of high levels of HLA-G [17]. These cells mainly act as regulatory cells, through the blocking the function of effector cells, and inducing the generation of suppressive cells.

However, whether peripheral MDSCs express HLA-G in patients infected with hepatitis B virus (HBV) remains unknown. In this study, the frequency of peripheral MDSCs (Lin1-HLA-DR-CD33+CD11b+) and HLA-G+ MDSCs subsets from HBV patients was analyzed. Our findings revealed that HLA-G+ MDSCs among peripheral blood monocytes (PBMC) were expanded in patients with chronic hepatitis B virus infection.

Materials and Methods

Study setting and participants

Consecutive blood samples were collected from 50 patients with CHB from the Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical College. The diagnosis was complied with the diagnostic criteria of the 2000 Xi'an Viral Hepatitis Management Scheme issued by the Chinese Society of Infectious Diseases and Parasitology, and the Chinese Society of Hepatology, of the Chinese Medical Association [25]. Briefly, a chronic hepatitis B patient is with clinical course of hepatitis B for >6 months and may have exhibited symptoms or signs of hepatitis and abnormal hepatic function on this occasion. No patients received anti-HBV agent or steroid 6 months before sampling. Subjects who infected or concurrence of HAV, HCV, HIV infections and other diseases were excluded. Samples from 27 healthy individuals without HBV infection history and with similar age and sex characteristics were included as controls. The study protocol was approved by the ethics committee of the Taizhou Hospital of Zhejiang Province, and informed consent was obtained from all of the subjects. The baseline characteristics of subjects enrolled in the study were shown in Table 1.

Variables	Controls	CHB						
	n=27	n=50	Correlate to parameters of CHB patients (p value)					
			MDSC	$CD14^+$MDSC	$CD15^+$MDSC	$HLA\text{-}G^+$ MDSC	$HLA\text{-}G^+$ $CD14^+$MDSC	$HLA\text{-}G^+$ $CD15^+$MDSC
Age (yrs)	45.6 ± 9.2	46.6.1 ±12.4	0.490	0.496	0.553	0.351	0.715	0.179
Sex (male/female)	16/11	38/12	0.237	0.215	0.031	0.771	0.140	0.307
HBsAg positive (%)	0	50 (100%)						
HBsAb positive (%)	0	1 (2.0%)					.	
HBeAg positive (%)	0	15 (30.0%)	0.072	0.534	0.150	0.808	0.643	0.244
HBeAb positive (%)	0	25 (50.0%)	0.088	0.498	0.077	0.843	0.279	0.048
HBcAb positive (%)	0	50 (100%)						
ALT (IU/L)	<40	340.5 ± 537.4	0.482	0.791	0.151	0.347	0.643	0.188
AST (IU/L)	-	170.7 ± 197.3	0.955	0.916	0.305	0.360	0.231	0.418
ALP(IU/L)	-	140.6 ± 52.4	0.609	0.380	0.091	0.027	0.952	0.438
GGT(IU/L)	-	119.0 ± 97.7	0.234	0.436	0.008	0.618	0.816	0.250
TBil (μmol/L)	-	47.1 ± 76.3	0.247	0.840	0.065	0.098	0.455	0.606
HBV DNA (log10)a	-	5.28 ± 2.04	0.833	0.371	0.063	0.665	0.295	0.032

CHB: Chronic hepatitis B patients; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Glutamyl transpeptidase; HBV: Hepatitis B virus; TBil: Total bilirubin; -: not detected.

Table 1: Baseline characteristics of the study populations.

Virological assessments

HBV serum markers were determined using commercial enzyme immunoassay kits (AXSYM System, Abbott, Wiesbaden, Germany). HBV DNA was extracted from serum samples and quantified using a commercial polymerase chain reaction (PCR) diagnostic kit with detection limit of 100 copies/ml (Sybio, Shanghai, China). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin concentration levels were measured by an auto biochemical analyzer (Olympus AU 5400, Olympus Corporation, Tokyo, Japan).

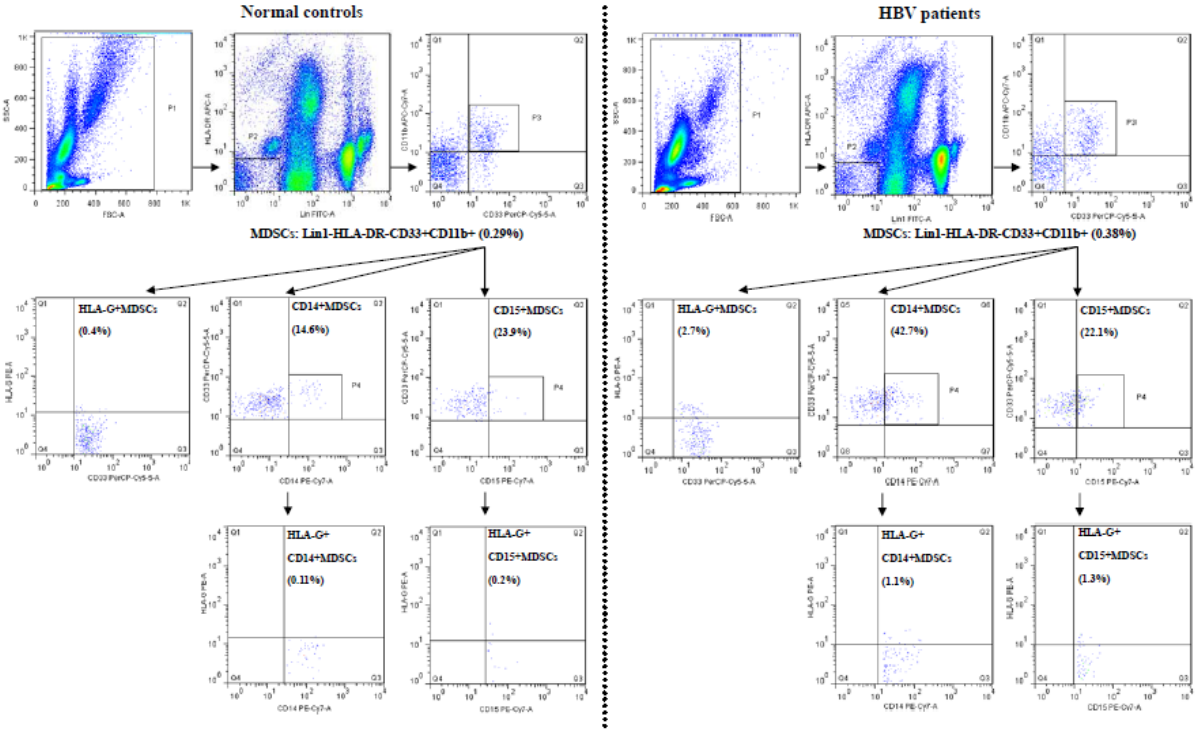

Figure 1: Phenotypic analysis and frequencies of MDSCs subsets in patients with chronic HBV infection and normal controls. Flow cytometric evaluation of Lin, HLA-DR, CD33, CD11b, HLA-G, CD15, and CD14 in PBMCs. An example of representative dot plots is shown for each group. Markers for MDSCs: Lin-HLA-DR-CD33$^+$CD11b$^+$, gMDSCs: Lin-HLA-DR- CD33$^+$CD11b$^+$CD15$^+$ and mMDSCs: Lin-HLA-DR-CD33$^+$CD11b$^+$CD14$^+$. Furthermore, HLA-G expression on MDSCs, gMDSCs and mMDSCs was analyzed. Gates were set based on appropriate isotype controls. The frequency of MDSCs defined as the percentage of MDSCs among total PBMC. The frequency of gMDSCs, mMDSCs and HLA-G expressing (MDSCs, gMDSCs and mMDSCs) defined as the percentage of respective MDSC subsets among total MDSCs. The type of each MDSC subset and frequency was presented in parenthese in each plot inside.

Whole blood staining and flow cytometric analysis

The flow cytometry analysis was performed with the protocol described in a previous study [26]. Briefly, 100 µL whole blood was taken for MDSC analysis. MDSC subsets were determined using the following antibodies: anti-lineage cocktail 1 (Lin1)-FITC, anti-HLA-DR-APC, anti-CD33-PercpCy5.5, anti-CD11b-APC-Cy7, anti-CD14-PE- Cy7, anti-CD15-PE- Cy7 (BD Pharmingen, San Jose, CA), and anti-HLA-G-PE (Exbio, Modrice, Czech Republic). Six-colour flow cytometric analysis was performed on a BD Canto II flow cytometer. Analysis of the flow cytometric data was performed using the BD FACSuite software. Sample tubes were gently vortexed and incubated for 25 min at 4. After the incubation, lysis buffer (BD FACS lysing solution) was added to lyse the red blood cells and then incubated for 15 min at room temperature. The samples were washed twice with phosphate buffered saline. The pellet was resuspended in 300 µL of flow cytometry buffer before analysis.

Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL). Normality of continuous numeric data was analyzed by one-sample Kolmogorov-Smirnov test. Comparison of the percentage of MDSCs and its subsets, HLA-G+ MDSCs between different groups was performed using the Mann-Whitney U test. The Spearman test was used to access the correlation between MDSCs subsets and clinical parameters. A two-sided p value<0.05 was considered as statistically significant.

Results

Frequency of total and subsets of MDSCs in patients with chronic HBV infection and normal controls

Herein, both total and subsets of circulating MDSCs from patients with chronic HBV infection and normal controls were analyzed using multicolour flow cytometry. We defined the MDSCs as Lin-HLA-DR-CD33$^+$CD11b$^+$, gMDSCs as Lin-HLA-DR- CD33$^+$CD11b$^+$CD15$^+$ and mMDSCs as Lin-HLA-DR-CD33$^+$CD11b$^+$CD14$^+$. Furthermore, HLA-G expression on MDSCs, gMDSCs and mMDSCs was analyzed.

The frequency of MDSCs defined as the percentage of MDSCs among PBMC. The frequency of gMDSCs, mMDSCs and HLA-G expressing (MDSCs, gMDSCs and mMDSCs) defined as the percentage of respective MDSC subsets among MDSCs. Representative flow cytometric plots of patients with chronic HBV infection and normal controls were shown in Figure 1.

The range of the frequency of MDSCs, gMDSCs, mMDSCs was 0.02%~1.06%, 2.60%~86.4% and 7.00%~71.50% in patients with chronic HBV infection, and 0.03%~0.74%, 5.40%~36.40% and 10.50%~61.10% in normal controls, respectively. Between patients with chronic HBV infection and normal controls, no significant difference was observed for the frequency of circulating MDSCs (median: 0.30% *vs.* 0.29%; p=0.884) (Figure 2A), subsets of mMDSCs (median: 31.25% *vs.* 23.35%; p=0.063) (Figure 2B) and gMDSCs (median: 22.60% *vs.* 21.55%; p=0.588) (Figure 2C).

Frequency of HLA-G expressing on both total and subsets of MDSCs was significantly elevated in patients with chronic HBV infection

Data showed that, the range of the frequency of HLA-G expressing MDSCs, gMDSCs, mMDSCs was 0.10%~18.90%, 0.00%~7.47% and 0.00%~10.92% in patients with chronic HBV infection, and 0.00%~6.20%, 0.00%~2.22% and 0.00%~2.44% in normal controls, respectively. All of these HLA-G expressing MDSCs was significantly higher in patients with chronic HBV infection compared with normal controls, where HLA-G+ MDSCs (median: 3.30% *vs.* 0.50%; p<0.001) (Figure 2D), HLA-G+ M-MDSCs (median: 0.99% *vs.* 0.00%; p<0.001 (Figure 2E) and HLA-G$^+$ gMDSCs (median: 0.78% *vs.* 0.00%; p<0.001) (Figure 2F).

Correlation of MDSCs subsets to clinical parameters in patients with chronic HBV infection

We then determined whether the frequency of MDSCs subsets in patients with chronic HBV infection was associated with their clinical parameters (Table 1). Interestingly, we found that the frequency of gMDSCs was significantly higher in male patients than that in female patients (median: 23.2% *vs.* 15.9%; p=0.031) (Figure 3A), which was also positively correlated with the levels of glutamyl transpeptidase (GGT) in HBV infected patients (r=0.372; p=0.008) (Figure 3B). The frequency of HLA-G+ gMDSCs much higher in HBeAb positive than that in HBeAb negative patients (median: 1.13% *vs.* 0.33%; p=0.048) (Figure 3C) and negatively associated with the HBV DNA copies (r=-0.310; p=0.032) (Figure 3D), and the frequency of HLA-G$^+$ MDSCs was inversely correlated with the levels of alkaline phosphatase (ALP) in HBV infected patients (r=-0.313; p=0.027) (Figure 3E).

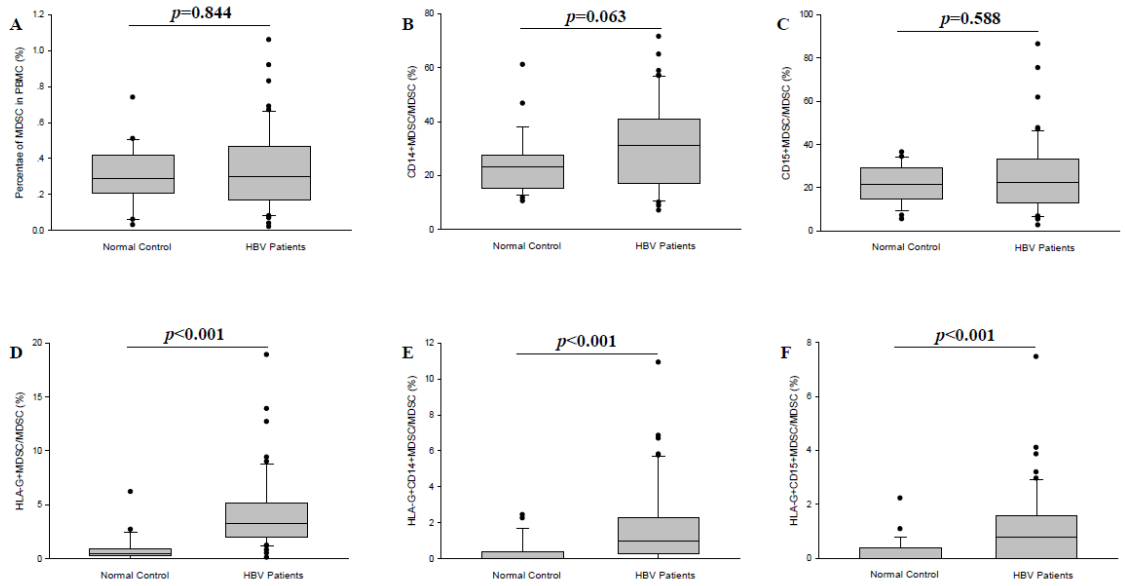

Figure 2: Comparison of the frequency of (A) MDSCs; (B) mMDSCs; (C) gMDSCs; (D) HLA-G$^+$ MDSCs; (E) HLA-G$^+$ mMDSCs and (F) HLA-G$^+$ gMDSCs between patients with chronic HBV infection and normal controls.

Discussion

MDSCs are a heterogeneous subsets of immature and progenitor myeloid cells that are characterized by their strong immunosuppressive ability, such as suppress NK and NKT cells, CD4$^+$ and CD8$^+$ T cells cytotoxic reactivity, modulate macrophages to an immunosuppressive M2 phenotype and induce the generation of regulatory T cells and regulatory DCs [6,27]. MDSCs have been extensively investigated and its clinical relevance was proposed in various malignancies in last two decades. However, the significance of MDSCs has been gaining its interesting in a number of viral infections [5,28].

The phenotype of murine MDSCs is CD11b$^+$Gr1$^+$, which according to the expression of Gr-1 recognized by antibodies to Ly6G and Ly6C, can be classified as either gMDSCs (CD11b$^+$Ly6G$^+$Ly6C^{low}) or mMDSCs (CD11b$^+$Ly6G$^-$Ly6^{Chi}), while cell markers for human MDSCs varies. Nevertheless, there is a growing consensus to define human MDSCs as Lin-CD11b$^+$CD33$^+$HLA$^-$DR$^{low/-}$. Among this population, the CD14$^+$CD15$^{low/-}$ subsets described as the human mMDSCs and CD14$^-$CD15$^+$ subsets described as the human gMDSCs [29].

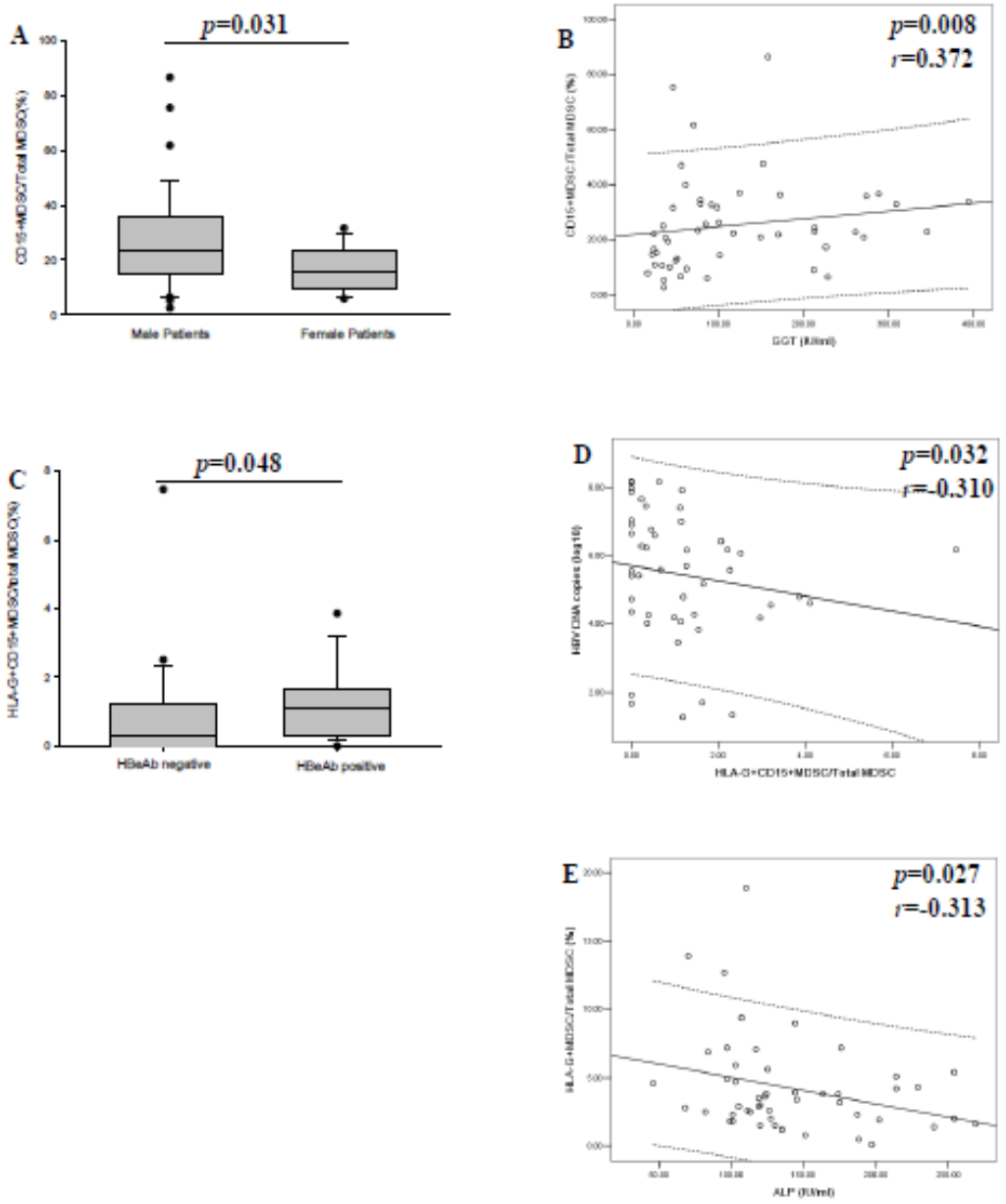

Figure 3: (A) Comparison of gMDSCs between male and female patients; (B) correlation between gMDSCs and levels of GGT; (C) comparison of HLA-G$^+$ gMDSCs between the HBeAb negative and positive patients; (D) correlation between HLA-G$^+$ gMDSCs and levels of HBV DNA copies and (E) between HLA-G$^+$ MDSCs and levels of ALP in HBV infected patients.

HLA-G is a nonclassical HLA class I molecule which plays immune suppressive functions in various physiological and pathological situations such as fetus and transplant acceptance, immune escaping of malignant and virus-infected cells [30]. The immune tolerant properties of HLA-G acts mainly through inhibitory receptors ILT2 and ILT4. As a consequence, HLA-G could directly inhibit the functions of NK cells, CTLs, B cells, neutrophils, DCs and MDSCs [31]. HLA-G has also indirect immune-regulatory activities by inducing tolerogenic cells including HLA-G-expressing APC, mesenchymal stem cells (MSCs), T regulatory (Treg) cells, CD4^{low} and CD8^{low} suppressor T cells, T regulatory type 1 (Tr1) cells, NK cells, and DC-10 cells [32,33]. Thus, HLA-G renders multiple effects in the suppression of immune responses.

In the context of MDSCs, interaction between HLA-G and murine cell receptor PIR-B (homology with the human ILTs) could expand the population of CD11^{b+}Gr^{1+}PIR-$^{B+}$ or CD11b$^+$ Ly6G$^+$ MDSCs, which could decrease NK cytotoxic activity [19,20]. Moreover, in ILT-2

transgenic mice, HLA-G has been found to induce the emergence of $CD11b^+$ $Gr1^+$ MDSCs with an enhanced suppressive activity and directly involved in the prolongation of allogeneic skin graft survival [34].

As for HBV infection, liver-derived MDSCs could suppress the proliferation of allogenic T cells and HBsAg-specific lymphocytes in an HBV transgenic murine model [35]. A recent finding revealed that HBsAg could promote differentiation of monocytes into M-MDSCs which could suppress HBV-specific T cell responses [36]. MDSC was also found to promote $CD8^+$ T cell exhaustion by γδT cells in HBV-carrier immunocompetent mice [37]. These studies indicated that MDSCs could provide an additional means for virus to escape from host immune clearance. However, whether either peripheral MDSCs express HLA-G or its clinical relevance in patients infected with HBV remains unknown.

In contrast to previous above-mentioned studies, our data revealed that peripheral MDSCs were not significantly increased in patients with chronic HBV infection when compared to normal controls. Among total MDSCs, also no significance was observed for the mMDSC and gMDSC between the two groups. Indeed, inconsistent results for MDSCs in various virus infections were not rare among previous studies [5]. A study by Nonnenmann et al. [38] reported that peripheral MDSCs were not significantly increased in patients with chronic HCV infection, and there was no difference in MDSC based on genotype or viral load, and a similar effect was found for suppression the function of $CD8^+$ T cells. In another study, mMDSCs were observed to be unrelated to HCV RNA loads, and levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) [39].

In this study, we found HLA-G^+ gMDSCs was negatively associated with the HBV DNA copies, and that was much higher in HBeAb positive than that in HBeAb negative patients. The data is reasonable because the viral replication suppression and viral load reduction was observed when loss of HBeAg after seroconversion by a reduced cccDNA load in the liver [40]. Also, HLA-G^+ MDSCs was inversely correlated with the levels of ALP, gMDSCs was positively correlated with the levels of GGT in HBV infected patients. The discrepancy might be explained by the differences in viral factors among different viral isolates or virally derived factors in generation and accumulation of MDSCs over the course of infection, or even by the influence of host genetic background and/or environmental differences. In this regard, Ning et al. [39] reported that the frequency of mMDSCs in HCV infected patients had certain relevance with age, where patients older than 40 years old group had a significantly higher frequency of mMDSCs than that of age less than 40 years old group. In our study, however, we found that the frequency of gMDSCs was significantly higher in male than that in female HBV infected patients. Recently, an important study by Pallett et al. [41] revealed that, during various HBV infected disease activity, gMDSC counts changes dynamically. In that study, authors found gMDSCs were only expanded and highest during virus replication without liver damage, while the number of gMDSCs declined prior to hepatic flares. Therefore, the variation of disease activity itself could also contribute the discrepancy.

Importantly, we for the first time found that HLA-G^+ MDSCs, and the subsets population of HLA-G^+ mMDSC and HLA-G^+ gMDSC were dramatically increased in patients with chronic HBV infection. The immune inhibitory roles of HLA-G-expressing cells such as APC, MSC, T cells, NK cells and DC-10 cells, have been well documented in a mount of studies [17,33]. In this scenario, LeMaoult et al. [42] addressed that HLA-G-expressing APCs could inhibit the proliferation of $CD4^+$ T cells, induce $CD4^+$ T cell anergy, and promote the differentiation of $CD4^+$ T cells into suppressive cells. Moreover, HLA-G molecules expressing on APCs could be shed or acquired by other cells through the process of trogocytosis, might provide extra inhibitory or proapoptotic signals. Thus, HLA-G^-expressing immune cells could suppress the function of effector cells directly, and/or induce the generation of suppressive cells, render multiple effects in the modulation of immune responses [31].

In summary, this work report for the first time that HLA-G^+ MDSCs, a new population of peripheral MDSCs, were expanded in patients with chronic hepatitis B virus infection. Particularly, HLA-G^+ gMDSC was inversely correlated to the viral DNA loads and significantly increased in HBeAb positive patients. Due to lack of functional analysis in this study, the potential immune roles of the HLA-G^+ MDSCs population remain to be elucidated.

Limitations of the Study

In this study, potential mechanisms underlining up-regulation of HLA-G-expressing MDSCs, and biological function and clinical relevance of HLA-G-expressing MDSCs during HBV infection was not investigated.

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