RESEARCH ARTICLE

Expression and Clinical Significance of Myeloid Derived Suppressor Cells in Chronic Hepatitis B Patients

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Abstract

We here document discovery of expression profile of myeloid derived suppressor cells (MDSCs) in chronic hepatitis B (CHB) patients and changes in the course of disease. The study population was composed of 75 outpatient HBV cases and 15 healthy control cases. Peripheral blood samples were collected for separation of mononuclear cells. Levels of MDSCs labeled with Lin-DR-CD11b+CD33+ obtained from peripheral blood mononuclear cells (PBMC), were revealed to have significant differences between the CHB and other groups. They were 0.414% for health control cases and 0.226% for CHB cases (Z=-2.356, *p*=0.0189). It also observed that the group of HBeAg positive cases had significant difference in MDSCs/ PBMC median (X^2=11.877, *p*=0.003), compared with group of HBeAg negative cases and the healthy control group. It suggested considerable MDSCs might be involved in HBeAg immune tolerance. In addition, negative correlations between MDSCs/PBMC and parameters of ALT, AST and TBil, while positive correlation between MDSCs/ PBMC and ALB parameter were found. Multiple comparisons between the four phases and health control phase again, there was a statistically sifnificant difference (X^2=17.198, *p*=0.002). Taken together, these findings may provide a new immunotherapy strategy for reduced the expression levels of MDSCs in CHB patients, through induction of an autoimmune response to virus removal.

Keywords: Myeloid derived suppressor cells - chronic hepatitis B - HBeAg

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Introduction

Although the vaccine could reduce the incidence of Hepatitis B widely, the Hepatitis B virus (HBV) infections and invasions remain a major global health problem. The World Health Organization repotted that there were about 20 million people had been infected by HBV, in which 3.5 million converted to chronic hepatitis and there were 0.7 million chronic hepatitis B patients had died of liver failure, cirrhosis and primary hepatocelluar carcinoma caused by HBV in 2013 (Lim et al., 2014). The pathogenesis of HBV persistent infection remains unclear until now. But it is clear that the depletion of specific T lymphocytes' immune function is an important feature with pathogenesis. And the inhibition microenviroment with various inhibitory receptors, inhibitory cells and immunosuppression cytokine accelerate the depletion (Crawford et al., 2009; Tinoco et al., 2009).

Myeloid-derived suppressor cells (MDSCs) could strongly inhibit the T cellular response, which was found as heterogeneity cell populations derived from bone marrow in the 1980s (Dmitry, 2009; Martin et al., 2012). While MDSCs have been characterized as co-expression of myeloid differentiation antigens GR-1 and CD11b cells in mice, MDSCs have been generally defined as linl-HLA-DR-CDllb+CD33+ cells in humans even though without uniform health standards (Peranzoni et al., 2010). MDSCs were first discovered in tumor tissue, which played a role in tumor metastasis, staging and immune evasion (Qu et al., 2012). It could make colorectal carcinoma peritoneal and systemic effectively metastases in peritoneum microenvironment by experiments between tumor immunized and non-immunized mice (Yu et al., 2013).

Recent studies have shown that MDSCs even presented in viral infectious diseases, and inhibited the T cell proliferation, E.g. hepatitis C Viral (HCV) and Acquired Immune Deficiency Syndrome (AIDS) (Tacke et al., 2012; Vollbrecht et al., 2012; Cai et al., 2013; Qin et al., 2013). On the other hand, complications of chronic hepatitis B (CHB), e.g. pancreatic cancer were on increasing concern. Doctor Li suggested that CHV infection may increase the risk of pancreatic cancer by meta-analysis (Li et al., 2013). However; MDSCs expression about the mechanism and course of the disease for chronic HBV patients or its complications was limited.

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In this study, the expression of MDSCs was detected by flow cytometry and correlation of clinical parameters was analyzed in CHB patients. An object thereof was defining the pathogenesis on CHB persistent infection and prerequisite for virus immunotherapy removal.

Materials and Methods

Survey and Respondents

The study population was composed of 75 outpatient HBV cases and 15 healthy control cases, which were from the third affiliated hospital, Sun Yat-Sen University (Guangzhou, China), from July 2012 to April 2013.

The 75 HBV cases were divided into three groups, which comprised with group one for 15 immune tolerance cases, group two for inactive HBsAg carrying cases and group three for untreated chronic Hepatitis B (CHB) cases, according to the hepatitis disease stage. The untreated chronic Hepatitis B (CHB) cases were included 28 HBeAg-positive and 17 HBeAg-negative.

The diagnostic criteria abided by European Association for the Study of the Liver, 2012 (EASL2012) standards (EASL. 2012).

All the subjects were excluded: other hepatitis or HIV infection, other causes of liver damage, autoimmune disorders and neoplasm. And the female respondents were not pregnant or lactating. In the research process, the cases were untreated with the intervention by antivirals, immunosuppressants or immunomodulators, at least 6 months.

Medical Ethics Committee of Sun Yat-Sen University approval was obtained and all involved patients had previously provided their written, informed consent to have their clinical and pathogenic information used for research.

Reagents

The lymphoprep were purchased from Norway Axis-Shield PoC (Oslo, Norway). All fluorescent antibodies (linl-FITC; HLA-DR-PerCP; CDllb-APC; CD33-PE) were purchased from Becton & Dickinson (BD) Phariningen Company (United States, San Diego). Human Regulatory T cell Staining Kit (CD4-FITC, CD25-APC, FoxP3-PE) were purchased from eBioscience Company (United States, San Diego).

Collection the Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation with lymphoprep. Firstly, it made equal mixture of peripheral venous blood (pooled with ethylenediaminetetraacetic acid (EDTA)) with Phosphate Buffered Saline (PBS). Then it was slowly dropped into lymphocyte separation medium at one fifth of the volume. After centrifuging at 2000rpm for 20 minutes at 25°C, the mononuclear cell layer were gathered. These Lymphocytes were washing ten minutes for tow times by five times the volume of PBS at 25°C. By the ending, made the lymphocytes suspensions were at 1×10^7 per milliliter cell concentration by re-suspended with Roswell Park Memorial Institute (RPMI)-1640, using Cell counts.

After the above process, each sample was kept as single-cell suspensions in 1ml PBS for flow cytometric analysis and HBV cases supernatant for antibody and cytokine production evaluation, respectively.

Flow cytometric analysis

PBMC samples from groups of immune tolerance cases; inactive HBsAg carrying cases; CHB cases and control cases were pooled, spun, and resuspended in 0.1ml of sterile PBS. Incubated with 0.02ml monoclonal antibodies (lin1-FITC; HLA-DR-PerCPCy(TM) 5.5; CDllb-PE and CD33-APC) for 30 minutes at 4°C, and then washed one times by 2 ml PBS at 4°C. Expression levels of MDSCs on these cells labeled with Lin-DR-CD11b+CD33+ were analyzed by flow cytometry. To assay the presence of MDSCs in PBMC (MDSCs/ PBMC, %) were calculated by software FlowJ, at the meantime, isotype matched antibodies controls were to prevent non-specific staining.

Statistical analysis of data

All data are represented as means±SD (±s) of three or more independent experiments. The data are changed into normal distribution with logarithm if the original data are positive skewness distribution. Comparision among the experimental groups, and the correlations between expression levels of MDSCs and chronic Hepatitis B (CHB) process as well as pathological features in various target organs were analysed. If the Data are homogenous, Analysis of variance, Student-Newman-Keulsa and Pearson's correlation will be used. If the data are not homogenous, Kruskal-Wallis, Games-Howell test, as well as spearman's correlation analysis will be used. All the analyses were carried out using the SPSS17.0 software (SPSS Inc, Chicago, IL, USA). Values less than 0.05 were considered to be statistically significant. And GraphPad Prism 5.01 software (La Jolla, USA) was used to plot the statistical diagram.

Results

Subject

All serum samples from 75 HBV cases and 15 healthy control cases were under the biochemical and immunological detection, which were listed the results on Table1. It showed that there were no statistically significant in gender and age among groups. HBV-DNA was at a higher concentration on the group of immune tolerance cases compared with others ($p<0.05$). The CHB group was at high levels of Liver function parameters, which included ALT; AST; TBIL; CHE and PTA($p<0.05$). Chronic hepatitis B made liver in the disease state, regardless of positive or negative with HBeAg.

Flow cytometric spectrum of MDSCs

Expression levels of MDSCs on these cells labeled with Lin-DR-CD11b+CD33+ were analyzed by flow cytometry. To assay the presence of MDSCs in PBMC (MDSCs/ PBMC, %) were calculated. Firstly, there were significant differences ($p<0.05$) between 45 CHB cases

Table 1. The Results of Biochemical and Immunological Detection for Each of Research Group Serum

Parameters	Healthy control cases (n=15)	Immune tolerance cases (n=15)	Chronic Hepatitis B (CHB) cases (n=45)		Inactive HBsAg carrying cases(n=15)
			HBeAg(+) (n=28)	HBeAg(-) (n=17)	
Age*	31.5±6.81	32.2±14.2	36.3±14	39.7±13	34.78±8.01
Gender#	8/7/14	8/7/14	28/13	12/17/14	9/6/14
HBV-DNA(log10U/ml)	ND	>7	6	5	<2
ALT(U/L)	ND	25.5	216	206	23
AST(U/L)	ND	22.5	87	94	23
TBIL(U/L)	ND	<23.9	81.3	35	<23.9
ALB g/l*	ND	>36.0	37.9±4.0	40.3±5.3	>36.0
CHE(U/L)*	ND	ND	5474.8±1901.4	6239.0±2812.7	ND
PTA (%)	ND	ND	12.9-15.4	12.9-15.6	ND

*Showed with X±S; #showed with Male to female ratio; ND mean Negative difference

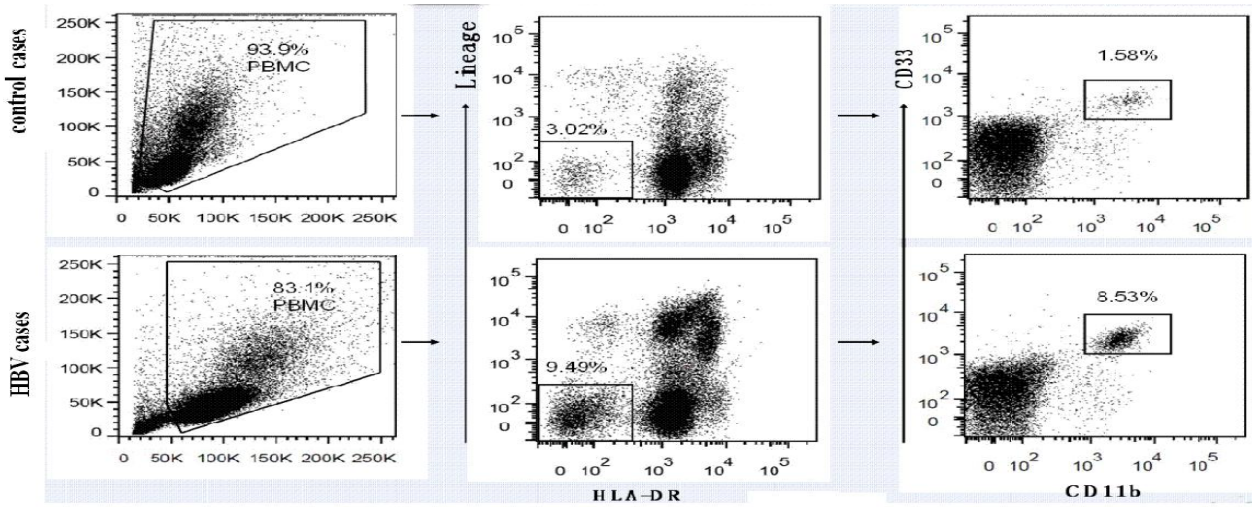

Figure 1. MDSCs were Highly Expressed in the PBMC of CHB Cases. There were significant differences ($p < 0.05$) between 45 CHB cases and 15 health control cases about Percentage of MDSCs/ PBMC

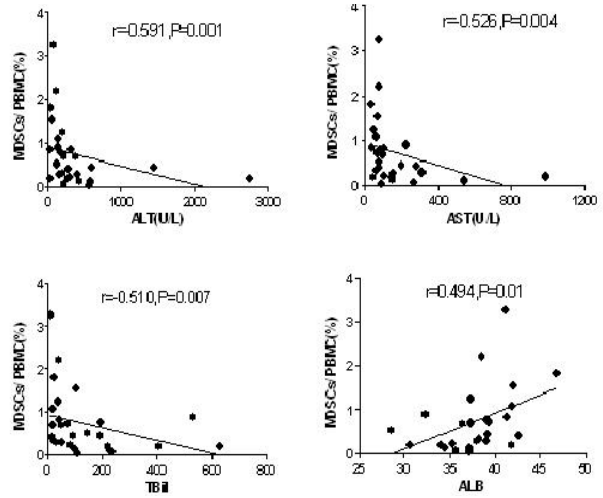

Figure 2. MDSCs Correlation Analysis with Liver FunctionIt found that Negative Correlations were between MDSCs/PBMC and Three Parameters (ALT; AST; TBil) and the Positive Correlation were between MDSCs/PBMC and One Parameter (ALB). It was regression analysis chart, which labeled the r and *p* values respectively

and 15 health control cases about Percentage of MDSCs/ PBMC, as showed in Figure 1. MDSCs were highly expressed in the PBMC of CHB cases. The median of MDSCs/ PBMC by spectrum value was further calculated, which were 0.414% for health control cases and 0.226% for CHB cases. A statistically significant difference was between them (Z=-2.356, p=0.0189).

In the more, the difference was concentrated in HBeAg positive cases, within the MDSCs/ PBMC median compared. The group of HBeAg positive cases had significant difference in MDSCs/ PBMC median (X^2=11.877, p=0.003), compared with group of HBeAg negative cases and health control group (By 28 HBeAg positive cases were with 0.493% and 17 HBeAg negative cases were with 0.183%).

MDSCs Correlation Analysis with Liver function and Viral loads

Correlation analyzed the MDSCs with Liver function parameters of 28 HBeAg positive cases detected by biochemical and immunological. It found that negative correlations were between MDSCs/ PBMC and three parameters (ALT; AST; TBil) and the positive correlation were between MDSCs/ PBMC and one parameter (ALB). Figure 2 was regression analysis chart, which labeled the r and p values respectively. Other two biochemical and immunological parameters (PT; CHE) had no correlation with MDSCs/ PBMC. On the other side, it also had no correlation with viral loads, which included parameters (HBV-DNA; HBs Ag; HBe Ag) (p>0.05).

It was further grouped for the HBeAg positive cases, referred to linear correlation parameters (ALT; AST;

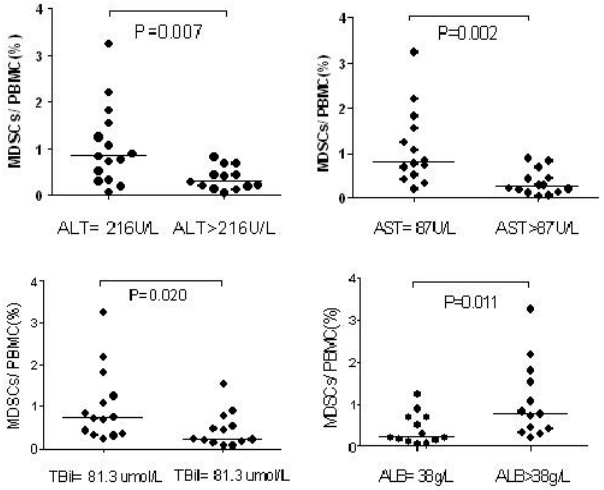

Figure 3. Linear Correlation with Parameters. The HBeAg positive cases group was on linear correlation parameters (ALT; AST; TBil and ALB)

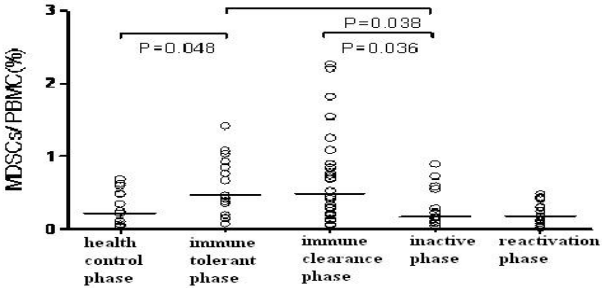

Figure 4. There was Statistical Difference between the Course of Disease and Health Control. The immune clearance phase was crucial phase about expression and clinical significance of myeloid derived suppressor cells in chronic hepatitis B patients

TBil and ALB). The results performed in Figure 3, which labeled *p* values respectively. So these were important detecting indicators for CHB.

Amount of MDSCs changes in the natural course of CHB

According to biochemical and immunological detection parameters and clinical symptoms, there were four phases as: immune tolerant phase; immune clearance phase (with HBeAg positive); inactive phase (only with HBs Ag positive) and reactivation phase (with HBeAg negative).

Multiple comparisons between the four phases and health control phase again, there were Statistical difference (X^2=17.198, p=0.002). As illustrated in Figure 4, the immune clearance phase was crucial phase about expression and clinical significance of myeloid derived suppressor cells in chronic hepatitis B patients.

Discussion

It has been reported that MDSCs presented in viral infectious diseases, autoimmune diseases, tumor, and parasitic infections diseases and inhibited the T cell proliferation (Greten et al., 2011; Tacke et al., 2012; Vollbrecht et al., 2012). However; MDSCs expression about the mechanism and course of the disease for chronic HBV patients was limited. In this study, the spectrum of MDSCs was at significantly high level in chronic hepatitis B patients' PBMC, compared with the health control cases. HBeAg was non-structural proteins of HBV, which didn't participate in packaging, infection and replication of HBV; it came into play with immunomodulatory effects in chronic hepatitis B patients yet.

Secreted proteins HBeAg were tolerogens, which were not only inhibit T helper 1-type (Th1) immune cells but also exhaust cytokines helped Th1 (Milich et al., 2003; Ito et al., 2009; Revill et al., 2010). But, it seemed very friendly with T helper 2-type (Th2) immune cells, activating the Th2 cells and promoting production of Th2 related cytokine.

Precisely these cytokine (including IL-6; IL-10; IL-13 and TGF-B) helped MDSCs gather and reproduce and another cluster cytokine (including IL-4; IL-13; IL-17and TGF-B) stimulated MDSCs activation (Gabrilovieh et al., 2009). By Doctor Yu research, a weak increase in IL-10 and a decrease of TGF-B was found in the lavage supernatant from tumor immunized group (Yu et al., 2013).

So, we speculated that there were difference of MDSCs expression between HBeAg positive cases and negative cases about CHB. Our experiments proved that. The group of HBeAg positive cases had significant difference in MDSCs/ PBMC median (X^2=11.877, p=0.003), compared with group of HBeAg negative cases and health control group. (By 28 HBeAg positive cases were with 0.493% and 17 HBeAg negative cases were with 0.183%).

While in the natural course of hepatitis B, it found that the expression of MDSCs decreased in CHB patients with seroconversion, even if there were reactivation or inflammatory lesions. These results suggested considerable MDSCs might involve in the HBeAg immune tolerance. The study of tumor showed that tumor-associated inflammatory cytokines help MDSCs gathering, activating and reproducing (Bunt et al., 2006).

In the study of hepatitis C, positive correlations were between MDSCs/ PBMC and tow parameters (AST and TBil). However in this study, it was negative correlations between them. It just was positive correlation with parameter of ALB. So, the high expression of MDSCs might happen to CHB at the earlier and lighter liver damage phase.

Currently, the nucleoside analogs and interferon were widely used to inhibit hepatitis B virus replication, reduce liver damage and retard disease progression. But they couldn't remove virus and covalently closed circular DNA (cccDNA).

The lamivudine (LVD) therapy has been commonly used in the treatment of CHB infections as a first line antiviral agent. But it shouldn't be ignored that the risk for developing drug-resistant mutations increases with duration of therapy. Although the research of viral gene mutation made considerable achievements, there was still a long term to targeted therapy of gene locus (Hakan et al., 2013).

The pathway of clearing the HBV and cccDNA might be by this way to achieve which recovery and improve the natural immune response and adaptive immune response, especially recovery the Th1 cells function

(Wang et al., 2009). MDSCs had been the target cells of Immunotherapy. E.g. gemcitabine could selectively remove the MDSCs (Suzuki et al., 2005); All Trans retinoic acid dihydroxyvitamin may induce differentiation and maturation of MDSCs (Lathers et al., 2004); nitro-Aspirin and Phosphodiesterase Inhibitors might Inhibitory activity of NOS and ARG, Avoiding serious adverse reactions (Serafini et al., 2006).

The study of MDSCs would not only disclose the pathogenesis of CHB persistent infection but also help immune intervention and therapy. In our study, it made MDSCs correlation analysis with liver function and viral loads, and analyzed amount of MDSCs changes in the natural course of CHB.

When the CHB patients had been detected with HBeAg positive, it recommended the use blocking or promoting differentiation agents for MDSCs. For example, Dihydroxyvitamin D3 had enhanced immune, enhanced CTL responses and promoted the virus removal, in our prospective study. But it still needs more experimental data from a large number of Participants. On the other hand, the Parameter index getting from biochemical and immunological detection might be helpful for diagnosis of CHB course and anti- MDSCs treatment. The inadequacies of this study were not implemented liver puncture and biopsy, and a limited number of specimens. More research is needed.

In summary, the expression of MDSCs was detected by flow cytometry and correlation of clinical parameters was analyzed in CHB patients. The Statistical difference was concentrated in HBeAg positive cases, within the MDSCs/ PBMC median compared. It found that negative correlations were with ALT; AST and TBil parameters, the positive correlation were with ALB parameter. The immune clearance phase was crucial phase about MDSCs expression and clinical significance. The study of MDSCs in CHB provided new thought precondition for Immunotherapy.

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References

Bunt SK, Sinha P, Clements VK, et al (2006). Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol*, **176**, 284-90.

Cai W, Qin A, Guo P, et al (2013). Clinical significance and functional studies of myeloid-derived suppressor cells in chronic hepatitis C patients. *J Clin Immunol*, **33**, 798-808.

Crawford A, Wherry EJ (2009). The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Curr Opin Immunol*, **21**, 179-86.

Dmitry I, Gabrilovich, Srinivas Nagaraj (2009). Myeloid-derived-suppressor cells as regulators of the immune system. *Nat Rev Immunol*, **9**, 162-74.

European Association for the Study of the Liver (2012). EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatology*, **57**, 167-85.

Gabrilovieh DI, Nagaraj S (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*, **9**, 162-74.

Greten TF, Manns MP, Korangy F,et al (2011). Myeloid derived suppressor cells in human diseases. *Int Immunopharmacol*, **11**, 802-7.

Hakan A, Ozturk O, Binnur K, et al (2013). Prevalence of common YMDD motif mutations in long term treated chronic HBV infections in a Turkish population. *Asian Pac J Cancer Prev*, 14, 5489-94.

Ito K, Kim KH, Lok AS, et a1 (2009). Characterization ofgenotype-specific carboxyl-terminal cleavage sites of hepatitis B virus e antigenprecursor and identification offurin as the candidate enzyme. *J Virol*, **83**, 3507-17.

Lathers DM, Clark JI, Achille NJ, et al (2004). Phase 1B study to improve immune responses in head and neck cancer patients using escalating doses of 25-hydroxyvitamin D3. *Cancer Immunol Immunother*, **53**, 422-30.

Li L, Wu B, Yang LB, et al (2013). Chronic hepatitis b virus infection and risk of pancreatic cancer: a meta-analysis. *Asian Pac J Cancer Prev*, **14**, 275-9.

Lim YS, Han S, Heo NY, et al (2014). Liver Transplantation, and Hepatocellular Carcinoma Among Patients with Chronic Hepatitis B Treated with Entecavir *vs* Lamivudine. *Gastroenterol*, **25**.

Martin F, Apetoh L, Ghiringhelli F (2012). Role of myeloid-derived suppressor cells in tumor immunotherapy. *Immunotherapy*, **4**, 43-57.

Milich D, Liang TJ (2003). Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology*, **38**, 1075-86.

Peranzoni E, Zilio S, Marigo I, et al (2010). Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol*, **22**, 238-44.

Qin A, Cai W, Pan T, et al (2013). Expansion of monocytic myeloid-derived suppressor cells dampens T cell function in HIV-1-seropositiveindividuals. *J Virol*, **87**, 1477-90.

Qu P, Boelte KC, Lin PC (2012). Negative regulation of myeloid-derived suppressor cells in cancer. *Immunol Invest*, **41**, 562-80.

Revill P, Yuen L, Walsh R, et a1 (2010). Bioinformatic analysis of the hepadnavirus e-antigen and its precursor identifies remarkable sequence conservation in all orthohepadnaviruses. *J Med Virol*, **82**, 104-15.

Serafini P, Borrello I, Bronte V, et al (2006). Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol*, **16**, 53-65.

Suzuki E, Kapoor V, Jassar AS, et al (2005). Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res*, **11**, 6713-21.

Tacke RS, Lee HC, Goh C, et al (2012). Myeloid suppressor cells induced by hepatitis C virus suppress T-cell responses through the production ofreactive oxygenspecies. *Hepatology*, **55**, 343-53.

Tinoco R, Alcalde V, Yang Y, et a1 (2009). Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+T cell deletion and viral persistence *in vivo*. *Immunity*, **31**, 145-57.

Vollbrecht T, Stirner R, Tufman A, et al (2012). Chronic progressive HIV-1 infection is associated with elevated levels of myeloid-derived suppressor cells. *AIDS*, **26**, 31-7.

Wang FS, Zhang Z (2009). Host immunity influences disease progression and antiviral efficacy in humansinfected with hepatitis B virus. *Expert Rev Gastroenterol Hepatol*, **3**, 499-512.

Yu M, Niu ZM, Wei YQ (2013). Effective response of the peritoneum microenvironment to peritoneal and systemic metastasis from colorectal carcinoma. *Asian Pac J Cancer Prev*, **14**, 7289-94.