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Myeloid-Derived Suppressor Cells Are Associated with Viral Persistence and Downregulation of TCR ζ Chain Expression on CD8$^+$ T Cells in Chronic Hepatitis C Patients

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Myeloid-derived suppressor cells (MDSCs) play an important role in impairing the function of T cells. We characterized MDSCs in two chronic hepatitis C (CHC) cohorts: a cross-sectional group that included 61 treatment-naive patients with CHC, 14 rapid virologic response (RVR) cases and 22 early virologic response (EVR) cases; and a longitudinal group of 13 cases of RVR and 10 cases of EVR after pegylated-interferon-α/ribavirin treatment for genotype 1b HCV infection. Liver samples from 32 CHC patients and six healthy controls were subjected to immunohistochemical analysis. MDSCs frequency in treatment-naive CHC was significantly higher than in RVR, EVR, or healthy subjects and was positively correlated with HCV RNA. Patients infected with HCV genotype 2a had a significantly higher frequency of MDSCs than those infected with genotype 1b. Decreased T cell receptor (TCR) ζ expression on CD8$^+$ T cells was significantly associated with an increased frequency of MDSCs in treatment-naive CHC patients and was restored by *L*-arginine treatment *in vitro*. Increased numbers of liver arginase-1$^+$ cells were closely associated with the histological activity index in CHC. The TCR ζ chain was significantly downregulated on hepatic CD8$^+$ T cells in CHC. During antiviral follow up, MDSCs frequency in peripheral blood mononuclear cells was directly correlated with the HCV RNA load in the plasma and inversely correlated with TCR ζ chain expression in CD8$^+$ T cells in both RVR and EVR cases. Notably, the RVR group had a higher frequency of MDSCs at baseline than the EVR group. Collectively, this study provides evidence that MDSCs might be associated with HCV persistence and downregulation of CD8 ζ chain expression.

INTRODUCTION

Most hepatitis C virus (HCV) infections in humans are associated with viral persistence, which leads to chronic hepatitis C (CHC); CHC affects more than 160 million people worldwide and is one of the main causes of liver cirrhosis and hepatocellular carcinoma (European Association of the Study of the Liver, 2012; Lavanchy, 2009; 2011). Protective immunological responses, including the vital role played by CD8$^+$ T cells during the control of HCV infection, are critical to viral clearance, (Neumann-Haefelin and Thimme, 2013). In patients with acute HCV infection, the HCV RNA load remains high during the first weeks of infection. However, a rapid decline in HCV RNA load is observed after 6-8 weeks, in parallel to the appearance of multi-specific and virus-specific CD8$^+$ T cells in the peripheral blood (Lechner et al., 2000; Neumann-Haefelin and Thimme, 2013; Thimme et al., 2001). In patients with CHC, HCV-specific CD8$^+$ T cell responses are typically weak and can often be lost due to viral escape mutations or suppression by regulatory T cells and the immune inhibitory pathway (Lauer, 2013; Neumann-Haefelin and Thimme, 2013). However, whether other suppressive mechanisms contribute to CD8$^+$ T cell dysfunction in CHC remains largely unclear.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous subset of immature myeloid cells (Almand et al., 2001; Bronte et al., 2000; Gabrilovich and Nagaraj, 2009; Young et al., 1987). Human MDSCs express the common myeloid markers CD11b and CD33 but lack HLA-DR and other surface markers for lymphoid cells, such as CD3, CD19 and CD56 (Filipazzi et al., 2007; Zea et al., 2005). Generally, MDSCs have been assigned an immune suppressive role against the host immunoprotective response to tumor cells, autoimmune disease and

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bacterial infections (Gabrilovich and Nagaraj, 2009). Mechanically, MDSCs suppress T cell responses *via* numerous mechanisms, including cysteine deprivation (Srivastava et al., 2010), up-regulation of reactive oxygen species (ROS) (Corzo et al., 2009; Tacke et al., 2012), production of nitric oxide in MDSCs (Bronte et al., 2003), induction of T regulatory cells (Hoechst et al., 2008), and increased metabolism of the amino acid L-arginine through the expression of arginase-1 (Ochoa et al., 2007); this latter pathway downregulates $CD3^+$ T cell receptor (TCR) ζ expression and inhibits T cell proliferation (Rodriguez et al., 2003).

Recent studies have suggested that MDSCs may play a role in the pathogenesis of viral infections (Chen et al., 2011; Macatangay et al., 2012; Qin et al., 2013; Vollbrecht et al., 2012). The frequency of MDSCs in the livers of hepatitis B virus (HBV) transgenic mice was significantly higher than in normal mice, and MDSCs suppressed the proliferative capacities of allogeneic T cells and hepatitis B virus surface antigen-specific lymphocytes through alteration of T cell antigens and impairment of interferon-γ production (Chen et al., 2011). In patients with human immunodeficiency virus (HIV) infection, elevated MDSC frequencies correlated positively with plasma HIV-1 viremia, and isolated MDSCs inhibited the proliferation of $CD8^+$ T cells *via* the induction of arginase-1 *in vitro* (Macatangay et al., 2012; Qin et al., 2013; Vollbrecht et al., 2012). Recently, Tacke and colleagues observed that HCV core antigen-treated $CD33^+$ MDSCs upregulated the expression of $p47^{phox}$, a component of the nitrogen oxide 2 complex that plays a pivotal role in ROS production. These *in vitro* data suggest that MDSCs induced by HCV suppress T cell responses, particularly through increasing the production of ROS (Tacke et al., 2012). Recently, emerging evidence has indicated that MDSCs may be implicated in driving liver disease progression by downregulating T cell function; however, the characteristics of the MDSCs in the liver of CHC patients remain unclear.

We hypothesize that MDSCs contribute to HCV persistence through the induction of arginase-1 to downregulate the expression of the TCR ζ chain and to suppress T cell proliferation *via* increased metabolism of the amino acid L-arginine. In this study, we characterized the MDSCs in two cohorts of patients to investigate the association between MDSCs and HCV persistence, as well as the downregulated T cell function, and the relationship between MDSC dynamics and the efficacy of antiviral therapy. In addition, we also investigated the characteristics of arginase-1 in the liver of patients with CHC and healthy controls, in order to provide information on the mechanisms underlying MDSC-involved HCV persistence.

MATERIALS AND METHODS

Subjects

Sixty-one treatment-naive patients with CHC, 14 patients with CHC undergoing pegylated-interferon-α/ribavirin therapy who developed a rapid virologic response (RVR) and 22 patients who developed an early virologic response (EVR) were enrolled in the cross-sectional group in this study. In addition, 23 treatment-naive patients with CHC who successfully completed a 12-week pegylated-interferon-α/ribavirin therapy follow-up study were enrolled in the longitudinal group. Liver samples from 32 voluntary patients with CHC and 6 healthy controls obtained from voluntary liver donors were used for immunohistochemical analysis. The degree of hepatic inflammation was graded from 0 to 4 according to the modified histological activity index (HAI) (Desmet et al., 1994); grading was used to describe the intensity of necroinflammatory activity. Patients with concurrent HBV or HIV infection, or autoimmune or alcoholic liver disease were excluded. The study protocol was approved by the Ethics Committee of our unit at Beijing 302 Hospital and informed consent was obtained from each subject. Baseline clinical data are shown in Table 1.

Flow cytometric analysis

All antibodies were purchased from BD Biosciences (USA) except for phycoerythrin (PE)-conjugated anti-CD33 (Pharmingen, USA), PE-conjugated TCR-ζ mAb (Beckman Coulter Immunotech SAS, France) and fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Sungene Biotech, China).

The peripheral blood cells were analyzed using protocols previously described by our team, with minor modifications (Zou et al., 2009). Anti-lin-1-FITC, anti-HLA-DR-peridin chlorophyll protein, anti-CD11b-allophycocyanin (APC), and anti-CD33-PE were used to characterize MDSCs. For phenotypic staining *ex vivo*, fresh heparinized peripheral blood (200 μl) was labeled with the above-mentioned cocktail of antibodies; matched isotype control antibodies were used as negative controls. After incubation for 30 min at 4°C in the dark, FACS™ lysing solution (BD PharMingen) was added to lyse the red blood cells. After washing twice with phosphate-buffered saline (PBS), the cells were fixed in 1% paraformaldehyde, and four-color flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, USA) and Flowjo software (TreeStar, USA) (Zhang et al., 2010). At least 200,000 events were acquired per run.

CD8 ζ chain detection

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml of heparinized blood by Ficoll-Hypaque density gradient centrifugation. Liver biopsy specimens were homogenized for isolation of liver-infiltrating lymphocytes (LILs) according to our previously described protocols (Xu et al., 2006). PBMCs and LILs were washed in PBS, resuspended in RPMI-1640 containing 10% fetal calf serum and then incubated at 4°C for 30 min with saturating concentrations of anti-CD3-FITC and anti-CD8-APC. Then, the cells were washed in PBS, fixed and permeabilized with cytoperm/cytofix for 30 min at 4°C before intracellular staining using anti-TCR-ζ-PE mAb or its corresponding isotype control IgG_1-PE mAb, and then analyzed using the FACSCalibur and FlowJo software.

Cell culture and CD8 ζ chain detection

Detection of CD8 ζ after culture in medium with or without L-arginine was performed according to previously described protocols (Das et al., 2008). PBMCs were incubated at 37°C overnight in either L-arginine-free medium or L-arginine-free medium supplemented with L-arginine (0.2 g/L, Sigma-Aldrich, USA). Then the cells were surface stained with anti-CD3-FITC and anti-CD8-APC and intracellularly stained with the anti-TCR ζ-PE mAb or its corresponding isotype control (IgG1-PE mAb), and finally analyzed using the FACSCalibur and FlowJo software.

Immunohistochemical staining

An anti-arginase-1 antibody for immunohistochemical staining was purchased from Beijing XinXingTang (China). Paraffin-embedded, formalin-fixed liver tissue (5 mm) sections were incubated with the anti-arginase-1 antibody overnight at 4°C after blocking endogenous peroxidase activity with 0.3% H_2O_2. Staining was developed using 3-amino-9-ethylcarbazole (red

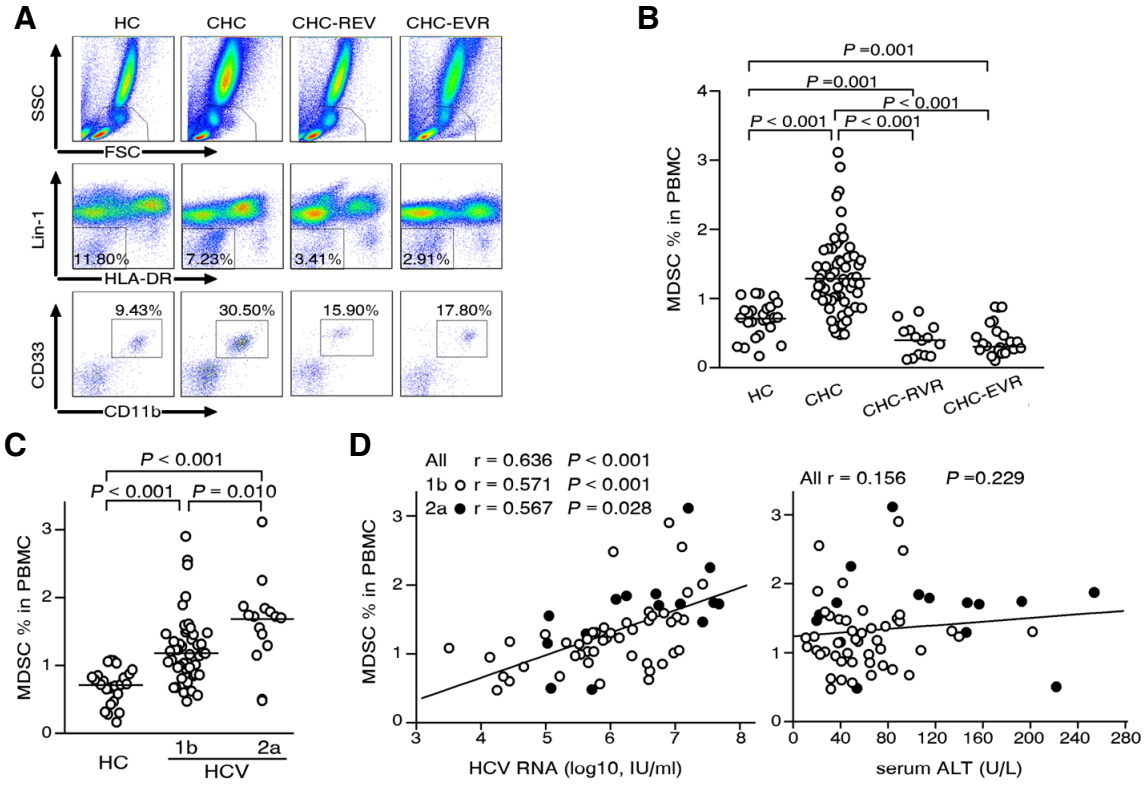

Fig. 1. The frequency of MDSCs increases and correlates with HCV RNA load in patients with chronic hepatitis C infection. (A) Representative dot plots; MDSCs were defined as the $CD33^+CD11b^+Lin1^-HLA\text{-}DR^-$ population; the frequency of circulating MDSCs was equal to the frequency of $Lin\text{-}1^-HLA\text{-}DR^-$ cells in PBMCs multiplied by the frequency of $CD33^+CD11b^+$ cells in $Lin\text{-}1^-HLA\text{-}DR^-$ cells. (B) Pooled data indicating the percentages of MDSCs in PBMCs from healthy controls (HC), patients with chronic hepatitis C (CHC), and patients with CHC with rapid virologic response (CHC-RVR) or early virologic response (CHC-EVR). The values in the quadrants indicate the percentages of the related subsets of cells. (C) Pooled data indicating the percentages of MDSCs in HC and in patients with CHC infected with HCV genotype 1b and genotype 2a. (D) The percentage of MDSCs in PBMCs correlated positively with the HCV RNA load in all patients with CHC, as well as the genotype 1b/2a subgroups (left). The percentage of MDSCs in PBMCs displayed no correlation with serum ALT levels in patients with CHC (right).

color) as a colorimetric substrate, followed by counterstaining with hematoxylin according to previously described protocols (Xu et al., 2006; Zhang et al., 2008; 2010). High-power fields (400×) were used for counting positive cells and the numbers of positive cells were counted in three different portal areas by two independent observers.

Virologic and immunological assessment

The levels of anti-HCV antibody were measured using the Ortho HCV ELISA kit and VITROS 3600 (Ortho Clinical Diagnostics, USA). HCV RNA levels were determined using a PCR Fluorescence Quantitative Diagnostic kit (Shanghai Kehua Bioengineering Co. Ltd, China) and Agilent StrataGene Mx3000P (USA). Both tests were performed according to the manufacturers' instructions. The threshold of detection for HCV RNA was 100 IU/ml.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 software for Windows (SPSS Inc. USA). Multiple comparisons were made between the different groups with the Kruskal-Wallis H nonparametric test. Comparisons between various groups were performed using the Mann-Whitney U test, whereas comparisons within the same group were performed with the Wilcoxon matched-pair *t*-test. Correlations between variables were evaluated with the Spearman rank correlation test. For all tests, a two-sided P value < 0.05 was considered significant.

RESULTS

Treatment-naive CHC patients have an increased frequency of MDSCs

To determine whether MDSCs play a role in the chronicity of HCV infection, we determined the frequency of MDSCs in the peripheral blood of patients with CHC. MDSCs were defined as the $CD33^+CD11b^+Lin1^-HLA\text{-}DR^-$ population (Fig. 1A). To identify MDSCs, we isolated PBMCs, excluded mature lymphocytes and the $HLA\text{-}DR^+$ population, and then used $CD33^+$ as a myeloid marker and CD11b as a functional marker. The frequency of circulating MDSCs was equal to the frequency of $Lin\text{-}1^-HLA\text{-}DR^-$ cells in PBMCs multiplied by the frequency of $CD33^+CD11b^+$ cells in $Lin\text{-}1^-HLA\text{-}DR^-$ cells. As expected, we observed a significantly higher frequency of MDSCs in treatment-naïve patients with CHC ($1.33 \pm 0.56\%$, n = 61) compared to healthy controls (HC; $0.70 \pm 0.26\%$, $P < 0.001$, n = 25), or patients with CHC-RVR ($0.50 \pm 0.37\%$, $P < 0.001$, n = 14) or CHC-EVR ($0.41 \pm 0.24\%$, $P < 0.001$, n = 22; Fig. 1B). Interestingly, we observed a significantly higher frequency of MDSCs in treat-

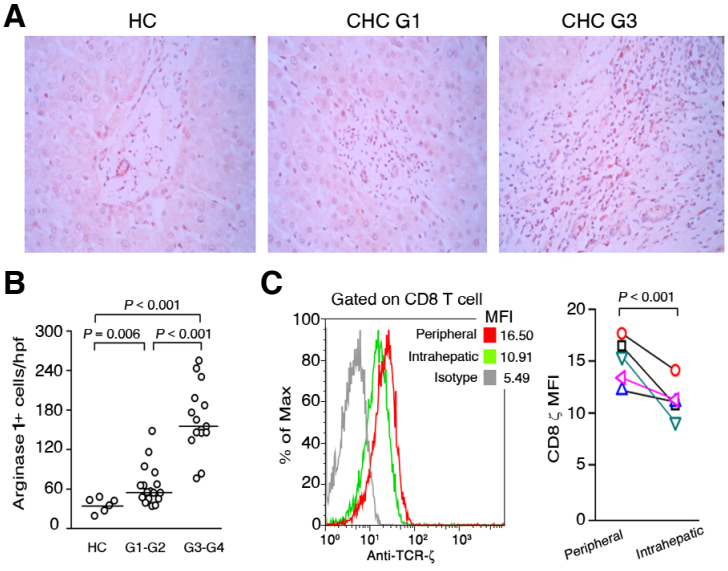

Fig. 2. The number of cells expressing arginase-1 increases and TCR ζ expression on CD8$^+$ T cells decreases in the livers of patients with chronic hepatitis C infection. (A) Immunohistochemical staining for arginase-1 in a portal area of the liver from a healthy control, and patients with CHC with HAI scores of G1 or G3 (400× magnification). (B) Pooled data indicating the numbers of arginase-1 positive cells in the portal areas of the liver in healthy controls, and patients with CHC with HAI scores of G1-2 and G3-4 (400× magnification). (C) Mean fluorescence intensity (MFI) values for CD8 ζ isolated from peripheral blood lymphocytes (PBLs) and liver tissues; the representative fluorescence-activated cell sorting (FACS) plots (left) and pooled data are shown (right).

ment-naïve patients with CHC compared to the CHC-RVR and CHC-EVR groups (both P = 0.001; Fig. 1B). Notably, patients infected with HCV genotype 2a had a significantly higher frequency of MDSCs than genotype 1b patients (P = 0.01); the significant differences in the frequency of MDSCs between HCs and patients with CHC were not affected by genotype (P < 0.001; Fig. 1C).

Next, we analyzed the correlation between the frequency of MDSCs and plasma HCV RNA load as well as serum ALT levels in treatment-naïve patients with CHC (Fig. 1D). There was a significant positive correlation between the frequency of MDSCs and HCV RNA load (Fig. 1D left, r = 0.636, P < 0.001). Further analysis indicated that these associations also existed in patients infected with genotype 1b (Fig. 1D left, r = 0.571 and P < 0.001) and genotype 2a (r = 0.567 and P = 0.028). However, no correlation was observed between the frequency of MDSCs and serum ALT levels in treatment-naïve patients with CHC (Fig. 1D right, r = 0.126, P = 0.334).

Increased numbers of arginase-1$^+$ cells are closely associated with the histological activity index in CHC

During viral infection, arginase-1, released from either MDSCs or damaged hepatocytes, is a central component of the cytokine milieu that accompanies both acute and chronic inflammation and viral disease (Das et al., 2008; Sandalova et al., 2012). We examined the distribution of arginase-1$^+$ cells in the liver of healthy individuals and patients with CHC. As shown in Fig. 2A (left), a small number of arginase-1$^+$ cells were observed in the portal areas of the liver in healthy individuals. Interestingly, increased numbers of arginase-1$^+$ cells were found to accumulate in the portal areas of the liver in patients with CHC (Fig. 2A middle and right). More importantly, arginase-1$^+$ cells were differentially distributed in patients with CHC with different HAI G scores; significantly more arginase-1$^+$ cells infiltrated the portal areas of patients with G3 or G4 scores compared to patients with G1 or G2 scores (Fig. 2B). Notably, the hepatocytes in the liver of patients with CHC and healthy individuals showed positive arginase-1$^+$ staining in cytoplasm. These data suggest that arginase-1$^+$ cells were markedly accumulated in the livers of patients with CHC, and the numbers of infiltrating arginase-1$^+$ cells are closely associated with the extent of inflammatory injury.

Arginase-1 produced by MDSCs is a major determinant of the suppression of CD8$^+$ T cell function through L-arginine depletion, which results in downregulation of CD3 ζ and inhibition of T cell function (Rodriguez and Ochoa, 2008). We investigated TCR ζ expression on CD8$^+$ T cells in LILs isolated from liver biopsy specimens, and also assessed TCR ζ expression on CD8$^+$ T cells in the liver and peripheral blood of patients with CHC. As presented in Fig. 2C (left), liver CD8$^+$ T cells had a lower TCR ζ mean fluorescence intensity (MFI) than CD8$^+$ T cells isolated from peripheral blood. In addition, pooled data showed that there was a significant trend towards a lower CD8 ζ MFI in CD8$^+$ T cells from LILs compared to peripheral blood CD8$^+$ T cells (Fig. 2C right, P < 0.001). These data suggest that hepatic TCR ζ expression on CD8$^+$ T cells might be downregulated by hepatic arginase-1 expression in patients with CHC.

Peripheral TCR ζ expression on CD8$^+$ T cells is downregulated and inversely correlates with the frequency of MDSCs at baseline in CHC

We detected peripheral TCR ζ expression on CD8$^+$ T cells using flow cytometry in healthy controls and patients with CHC, CHC-RVR and CHC-EVR. Representative TCR ζ expression on CD8$^+$ T cells after gating is shown for a patient with CHC and healthy donor in Fig. 3A. The CD8 ζ levels were significantly lower in patients with CHC at baseline than patients with CHC-RVR (P = 0.016), CHC-EVR (P < 0.001), or healthy controls (P < 0.001; Fig. 3B). Moreover, in patients with CHC, TCR ζ expression on CD8$^+$ T cells correlated inversely with the frequency of MDSCs at baseline (Fig. 3C, r = 0.690, P < 0.001).

Because CD8 ζ can be downregulated *via* depletion of L-arginine (Das et al., 2008), we investigated whether supplementation with L-arginine had the potential to upregulate TCR ζ expression on CD8$^+$ T cells. Compared to the levels immediately after *ex vivo* purification, the intracellular levels of ζ chain in CD8$^+$ T cells purified from patients with CHC were upregulated after overnight culture in complete L-arginine-free medium (Fig. 3D, P < 0.001). However, when L-arginine was added back to the medium, TCR ζ expression on CD8$^+$ T cells further increa-

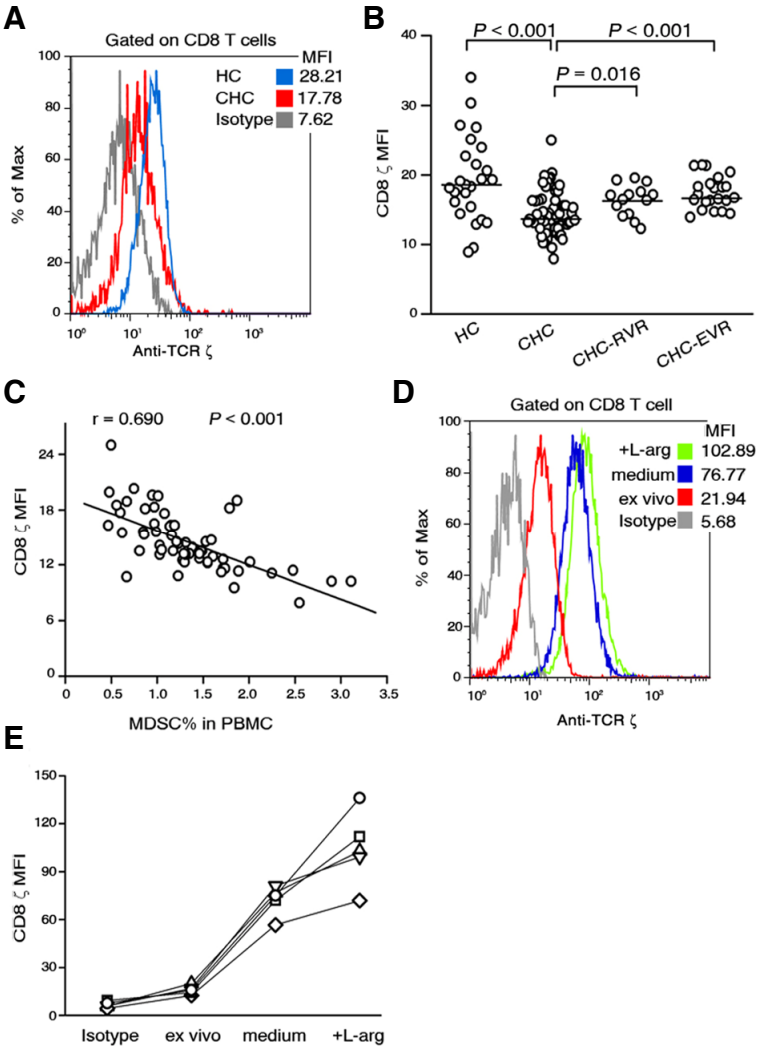

Fig. 3. Downregulation of TCR ζ expression on CD8$^+$ T cells inversely correlates with the frequency of MDSCs in patients with chronic hepatitis C and is upregulated by L-arginine *in vitro*. (A) FACS analysis of TCR ζ expression by CD8$^+$ T cells isolated from peripheral blood cells of healthy controls (HC), patients with CHC, and the CHC-RVR and CHC-EVR groups. Representative FACS analyses from the HCs and CHC groups are shown. (B) Pooled data indicating the mean fluorescence intensity (MFI) of TCR ζ expression on CD8$^+$ T cells in HCs, CHC and the CHC-RVR and CHC-EVR groups. (C) CD8 ζ MFI values inversely correlated with the percentage of MDSCs in PBMCs isolated from patients with CHC. (D, E) CD8 ζ on CD8$^+$ T cells was upregulated by treatment with L-arginine *in vitro*; representative FACS analyses (D) and pooled data (E) for CD8 ζ MFI values of PBMCs cultured in control medium or medium supplemented with L-arginine.

sed compared to CD8$^+$ T cells cultured in L-arginine-free medium (Figs. 3D and 3E, P < 0.001).

A decreased frequency of MDSCs is associated with restoration of TCR ζ expression on CD8$^+$ T cells in the RVR and EVR groups during antiviral therapy follow up

Twenty-three treatment-naive patients with CHC successfully completed a 12 week follow-up study, in which 13 patients acquired RVR and 10 patients acquired EVR. Based on the serum ALT levels and HCV RNA load, patients who achieved RVR showed the typical HCV kinetics, with undetectable plasma HCV RNA (< 100 IU/ml) at week 4 of therapy, whereas patients who achieved EVR displayed a complete absence of serum HCV RNA at week 12 of therapy (Fig. 4A). However, the serum ALT levels were not significantly different between the RVR and EVR groups at baseline and weeks 2, 4 and 12 of follow up (Fig. 4B).

We then longitudinally investigated the frequency of MDSCs and expression of CD8 ζ in the RVR and EVR groups. As shown in Fig. 4C, the frequency of MDSCs was significantly higher in the RVR group at baseline than the EVR group at baseline (P = 0.024). In addition, the frequency of MDSCs were considerably lower in both the EVR group and RVR group during treatment than at baseline (all P < 0.001); however, the frequencies of MDSCs in the RVR and EVR groups were similar at week 2, 4 and 12 (all P > 0.05).

As shown in Fig. 4D, compared to baseline, the MFI of CD8 ζ increased significantly in the RVR and EVR groups at weeks 4 and 12 (both P < 0.001), and the CD8 ζ MFI was similar in the RVR and EVR groups at weeks 4 and 12 (all P > 0.05). These results indicate that a decreased frequency of MDSCs positively correlates with the HCV RNA load and negatively correlates with TCR ζ expression on CD8$^+$ T cells in patients with CHC who achieved either RVR or EVR.

DISCUSSION

MDSCs are immunoregulatory cells that exhibit suppressive activities towards adaptive immunity (Kong et al., 2013; Tacke et al., 2012; Yang et al., 2012). However, the characteristics of MDSCs in patients with CHC remain unclear. Therefore, it is clinically relevant to investigate the association of the characteristics of MDSCs with HCV persistence, liver inflammation and antiviral efficacy in patients with CHC. In this study, we ob-

Table 1. Clinical characteristics of the populations enrolled in the study

Groups (cross-sectional)	Cases	Sex (male/female)	Age (years)	ALT (U/L)	HCV RNA (IU/ml)	HCV genotype (1b/2a)
Healthy controls	25	18/9	28(20-35)	Normal	Undetectable	NA
Chronic hepatitis C	61	38/23	45(19-70)	72(11-254)	5974541(3220-47980000)	46/15
CHC- RVR	14	10/4	40(19-56)	22(10-40)	Undetectable	9/5
CHC-EVR	22	13/9	42(20-63)	31(7-106)	Undetectable	17/5
Follow up group at baseline						
RVR	13	10/3	39(19-56)	86(27-193)	14965632(22220-48000000)	13/0
EVR	10	6/4	41(20-62)	74(27-202)	3709542(27660-11570000)	10/0

Abbreviations: CHC, chronic hepatitis C; EVR, early virologic response; NA, not applicable; RVR, rapid virologic response

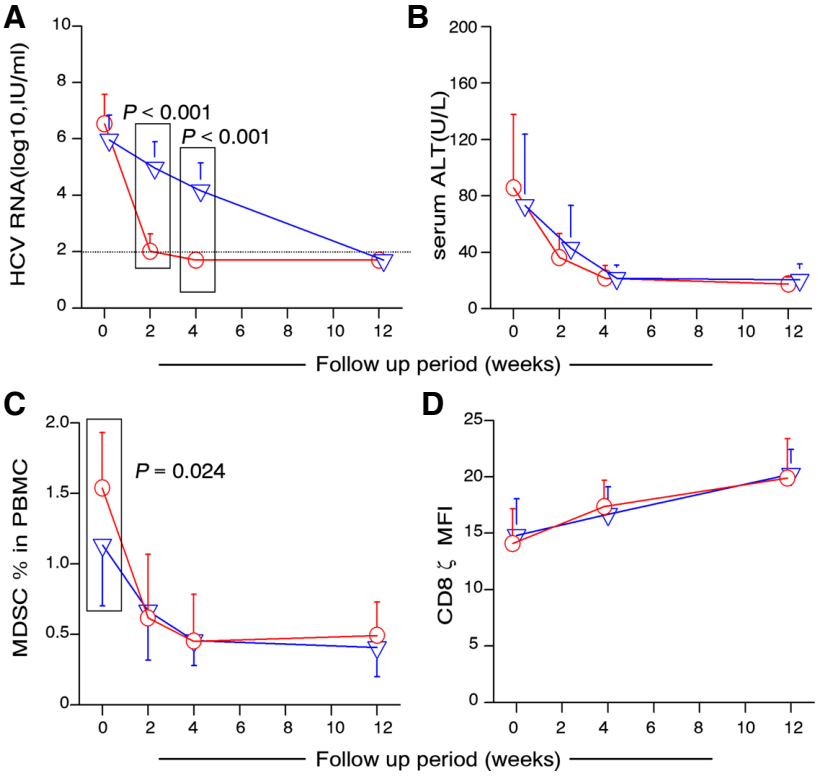

Fig. 4. HCV RNA load, serum ALT levels, frequency of MDSCs, and TCR ζ expression on $CD8^+$ T cells during antiviral therapy in patients with CHC who achieved RVR or EVR. (A) Serum HCV RNA levels during antiviral therapy in the RVR and EVR groups. Serum HCV RNA load was significantly lower at weeks 2, 4 and 12 compared with baseline in both groups, and significant differences were observed at weeks 2 and 4 between the two groups. (B) Serum ALT levels during antiviral therapy in the RVR and EVR groups. Serum ALT levels significantly decreased at weeks 2, 4 and 12 compared with baseline in both groups; no significant differences were observed between the two groups. (C) Percentage of MDSCs in PBMCs during antiviral therapy in the RVR and EVR groups. The percentages of MDSCs were significantly lower at weeks 2, 4 and 12 compared with baseline in both groups; the RVR group showed a significantly higher percentage of MDSCs at baseline than the EVR group at baseline. (D) Expression of TCR ζ by $CD8^+$ T cells during antiviral therapy in the RVR and EVR groups. The MFI for CD8 ζ was significantly higher at weeks 4 and 12 compared with baseline in both groups, no significant differences were observed between the two groups.

served that an increased frequency of MDSCs contributed to viral persistence, whereas a decreased frequency of MDSCs was associated with virologic response during pegylated-interferon-α/ribavirin treatment. In addition, we found that an increased frequency of MDSCs was closely associated with HCV genotype-2a infection, the HCV-RNA load, and the liver HAI G score, and patients infected with genotype-2a HCV displayed a tendency towards RVR during pegylated-interferon-α/ribavirin treatment.

During the standard treatment for CHC infection, three types of virologic response are used to evaluate short-term outcomes. Achievement of an RVR, which is defined as undetectable plasma HCV RNA (< 100 IU/ml) at week 4 of therapy, predicts a high likelihood of achieving a sustained virologic response (SVR), which is defined as undetectable plasma HCV RNA (< 100 IU/ml) at 24 weeks after the cessation of therapy (European Association of the Study of the Liver, 2012). An EVR is defined as a reduction (≥ 2 log) or complete absence of serum HCV RNA at week 12 of therapy compared with the baseline level. Failure to achieve EVR is regarded as the most accurate predictor of failure to achieve SVR (European Association of the Study of the Liver, 2012). In the present study, we hypothesized that MDSCs actively participate in regulating the antiviral responses in patients with CHC and that successful antiviral therapy would be associated with a decreased frequency of MDSCs.

The phenotypic markers of MDSCs vary between mice and humans. In the mouse, the MDSC populations include two groups: polymorphonuclear MDSCs described as $CD11b^+Gr\text{-}1^{high}Ly6G^+Ly6C^{low/int}$ cells, and mononuclear MDSCs described as $CD11b^+Gr\text{-}1^{int}Ly6G^+Ly6C^{high}$ cells (Condamine and Gabrilovich, 2011). However, the phenotypic markers of MDSCs are

less well characterized in humans. In humans, MDSCs are most commonly defined as $CD14^-CD11b^+$ cells, or more narrowly, as cells that express the common myeloid marker CD33 but lack the expression of markers of mature myeloid and lymphoid cells and HLA-DR (Gabrilovich and Nagaraj, 2009). Most studies have concluded that the human MDSC phenotype is $Lin^-HLA\text{-}DR^-CD33^+$ or $CD11b^+CD14^-CD33^+$ (Almand et al., 2001; Filipazzi et al., 2012; Gabrilovich and Nagarai, 2009; Kotsakis et al., 2012; Ramachandran et al., 2013; Vasquez-Dunddel et al., 2013); we utilized $Lin1^-HLA\text{-}DR^-CD33^+CD11b^+$ as markers of MDSCs in this study.

Recent studies indicated that MDSCs are involved in chronic HIV and HCV infection (Cai et al., 2013; Vollbrecht et al., 2012). For example, during HIV-1 infection, the characteristics of MDSCs have been reported to be closely associated with disease progression and the efficacy of highly active antiretroviral therapy (HAART). In treatment-naive patients with HIV, the frequency of MDSCs increases as clinical markers of HIV viral load and the CD4 T cell count increase. In addition, a decreased frequency of MDSCs is associated an increased effectiveness of HAART. In contrast, the characteristics of MDSCs in patients with CHC remain unclear. In the present study, we characterized MDSCs in two cohorts of patients with CHC, and found that the frequency of MDSCs was closely associated with HCV persistence, the degree of liver inflammation, and the efficacy of antiviral therapy. Firstly, the frequencies of MDSCs in treatment-naive patients with CHC were significantly higher than patients who achieved RVR or EVR, or healthy subjects. In addition, our results agreed with a previous report that an increased frequency of MDSCs positively correlated with the HCV RNA load (Cai et al., 2013). Secondly, MDSCs in an inflammatory environment produce arginase-1, and we found that increased numbers of arginase-1^+ cells in the liver were closely associated with the HAI in patients with CHC. Thirdly, the frequency of MDSCs in patients who achieved either EVR or RVR decreased with the HCV RNA load during antiviral therapy. Furthermore, detailed analysis found that an increased frequency of MDSCs was closely associated with HCV genotype-2a infection, and patients with a higher frequency of MDSCs showed a significant tendency for achieving RVR during pegylated-interferon-α/ribavirin treatment. In the clinic, acquisition of RVR during HCV genotype-2a infection can predict a higher likelihood of achieving SVR (European Association of the Study of the Liver, 2012), and taken together, these data indicate that MDSCs have the potential to predict disease progression and the efficacy of antiviral treatment in CHC.

Multiple mechanisms have been suggested to be implicated in the suppressive activity of MDSCs. In contrast, only limited information is available regarding how human MDSCs exert their suppressive function. The metabolism of L-arginine by MDSCs, which mediates T cell suppression through downregulating expression of the TCR ζ chain, is one of the most important mechanisms in both mice and humans. In the present study, we examined TCR ζ expression on $CD8^+$ T cells to assess the function of MDSCs in different disease stages. We found that both intrahepatic and peripheral TCR ζ expression on $CD8^+$ T cells decreased in patients with CHC compared to healthy controls. In addition, decreased peripheral TCR ζ expression on $CD8^+$ T cells inversely correlated with the frequency of MDSCs. Furthermore, decreased TCR ζ expression on $CD8^+$ T cells could be restored *in vitro* by treatment with L-arginine. Importantly, in patients who achieved RVR or EVR, the restoration of TCR ζ expression on $CD8^+$ T cells was closely associated with a decreased frequency of MDSCs. These data suggest that MDSCs participate in the entire pathologic process of HCV infection. However, recent studies have demonstrated that MDSCs mainly exert immunosuppressive functions through the induction of arginase-1 and L-arginine depletion (Kong et al., 2013; Rodriguez and Ochoa, 2008; Sandalova et al., 2012), whereas another report indicated that ROS-mediated suppression of T cell responsiveness plays an important role in the immunosuppressive function of MDSCs (Tacke et al., 2012). It will be of interest to elucidate the precise mechanisms by which MDSCs selectively facilitate arginase-1-mediated-, ROS-mediated- or a combination of arginase-1- and ROS-mediated immunosuppressive functions.

In summary, this study provides evidence associating the characteristics of MDSCs with HCV persistence through down-regulation of ζ chain expression on $CD8^+$ T cells in patients with CHC. These findings suggest a mechanism by which MDSCs may influence the function of $CD8^+$ T cells and also support the notion that MDSCs can be used as an immunological marker of disease progression and the efficacy of antiviral therapy in patients with chronic HCV infection.

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