Hepatitis C virus alternate reading frame protein decreases interferon-α secretion in peripheral blood mononuclear cells

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Abstract. The hepatitis C virus (HCV) alternate reading frame protein (ARFP or F protein) of the HCV 1b genotype is a double-frameshift product of the HCV core protein (Core). The discovery of HCV F protein challenges various biological functions attributed to Core. However, the specific characteristics of the host cellular immune response to F protein during HCV infection have yet to be fully elucidated. Therefore, the present study investigated the cytokine response to HCV Core or F protein in peripheral blood mononuclear cells (PBMCs) and plasmacytoid dendritic cells (PDCs) from patients with chronic HCV and healthy donors *in vitro*. The results demonstrated that the levels of interferon (IFN)-α, analyzed by an enzyme-linked immunosorbent assay, secreted by PBMCs in patients positive for the anti-F protein antibody, were lower than those of patients negative for the anti-F protein antibody. Moreover, the frequency of PDCs in patients negative for the anti-F protein antibody, were higher than in the group positive for the anti-F protein antibody. Furthermore, HCV F protein and Core not only inhibited specific unmethylated CpG oligonucleotide sequences of type A (CpG-A)-induced IFN-α production by PBMCs and PDCs, but also upregulated the production of interleukin (IL)-10 by PBMCs in patients with chronic HCV and healthy controls. Notably, following neutralization of IL-10 in the media and *in vitro* Core or F protein stimulation, levels of IFN-α were increased. Moreover, the results revealed that the roles of F protein and Core were similar with regard to the induction of apoptosis of PDCs in patients with chronic HCV. These findings suggest that F protein may inhibit PBMC IFN-α secretion by regulating the production of IL-10, and may contribute to an increase in the rates of apoptosis in PDCs. In conclusion, the results have revealed a potential involvement of F protein in the mechanisms of chronic hepatitis C.

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Introduction

The hepatitis C virus (HCV) is an important factor in the development of chronic hepatitis. A total of >170 million individuals are infected worldwide, among whom 80% develop chronic hepatitis (1,2). Chronic hepatitis C infection often results in cirrhosis, leading to hepatocellular carcinoma (HCC) within two to three decades of infection. To date, no effective vaccine is available, due to the extreme heterogeneity within the HCV genome, caused by the error-prone HCV RNA-dependent RNA polymerase (3,4).

HCV, a positive-sense and single-strand RNA virus, is a member of the Flaviviridae family and has a genome of ~9.6 kb in length (5). The genome is composed of an open reading frame (ORF) encoding a poly-protein of ~3,000 amino acids that is flanked by 5' and 3' non-coding regions. This polyprotein is cleaved into smaller proteins, including HCV core protein (Core), E1, E2, p7, NS2, NS3, NS4a, NS4b, NS5a and NS5b, by a combination of host and viral proteinases (6). According to previous studies, a novel core protein, known as alternate reading frame protein (ARFP or F protein), may be synthesized by ORF shift (7,8). The HCV 1b subtype is the most prevalent in China, and the HCV F protein is a double-frame shift product of the HCV core gene (9-11). The shift junction

in genotype HCV 1b generates a protein commencing with the initial 42 amino acids of Core, followed by 101 amino acids encoded in the ARF, followed by the C-terminus of Core (7,8). It has been demonstrated that the HCV F protein is not only expressed through a ribosomal frameshift within the core coding sequence of all HCV genotypes, but also demonstrates high variability among different HCV isolates.

It is well established that Core is involved in virion assembly, RNA replication, promotion and repression of cellular apoptosis, modulation of cytokine signaling and the cellular immune response (12,13). However, unlike Core, the HCV F protein does not appear to have a significant effect on HCV replication or the regulation of certain proto- or anti-oncogenes, such as c-myc and p53 (14,15). By contrast, previous studies have reported that the HCV F protein has a similar role to Core in inhibiting p21 expression, and promoting HCV infection by inducing secretion of the proinflammatory cytokine interleukin (IL)-6 (16,17). Moreover, antibodies and T cell-mediated immune responses specific to F protein have been detected in sera of patients infected with HCV, suggesting that F protein expression during HCV infection may have a role in the persistence of infection (17). Furthermore, cytokines present in the microenvironment have been shown to be important in viral clearance and persistence (18). Interferon (IFN)-α, largely sourced from plasmacytoid dendritic cells (PDCs), was found to be important in defense against HCV infection, through inhibition of viral replication, promotion of T cell survival and B cell antibody generation (19). Numerous previous studies comparing the production of IFN-α by PDCs between patients with chronic HCV and healthy individuals suggested that a reduction in IFN-α production may contribute to chronic hepatitis (20,21). In addition, accumulating evidence has indicated that Core causes PDC dysfunction and an imbalance of $CD4^+$ and $CD8^+$ T cells by increasing levels of IL-10, reducing the generation of IFN-α and inducing apoptosis in PDCs (22). However, whether the HCV F protein expressed by the core coding sequence has a similar effect to Core, with regard to reducing IFN-α production in patients infected with HCV, has yet to be elucidated.

Previous studies have not been able to exclude the influence of F protein on the specific characteristics of the host cellular immune response; therefore, the discovery of F protein challenges various activities attributed to HCV Core. In order to evaluate the host cellular immune response to Core or F protein, cytokine secretion and the functional properties of F protein and Core were analyzed in PBMCs and PDCs derived from human peripheral blood.

Materials and methods

Patients. Sixty treatment-naive patients with HCV infection (positive for HCV antibodies and negative for hepatitis B surface antigen) were enrolled in the study from blood donors in Jurong (China) between June and December 2012. All patients were diagnosed with chronic HCV infection which was confirmed as positive for anti-HCV in sera by third-generation ELISA kits and HCV RNA tests, with persistently elevated ALT levels for more than 2 years. Liver biopsy and ultrasound showed no fibrosis. No patients were co-infected with any other hepatotropic virus or showed any evidence of other causes of hepatocellular injury, such as drugs, alcohol, autoimmune or metabolic disorders (23). Anti-HCV antibodies were analyzed using third-generation HCV enzyme immunoassays (Shanghai Kehua Bio-engineering Co., Ltd, Shanghai, China). HCV genotypes were detected using polymerase chain reaction (PCR; Realchip Biotechnology Co., Ltd., Ningbo, China). Samples from 60 healthy, HCV-negative volunteers who were also paid blood donors were collected as healthy controls. The clinical characteristics of the patients are shown in Table I. Ethics approval was acquired from the Human Investigation Committee of the Huadong Research Institute for Medicine and Biotechnics (Nanjing, China). Furthermore, informed consent was obtained from all subjects prior to enrollment in the study.

Table I. Clinical characteristics of patients with HCV infection compared with healthy controls.

Characteristic	Chronic HCV	Healthy control
No. of patients	60	60
Age, years (mean ± SD)	56.64±7.59	56.26±7.50
Gender (male:female)	37:23	29:31
ALT (IU) (mean ± SD)	47.22±32.20	21.93±7.99
AST (IU) (mean ± SD)	45.83±33.24	24.73±7.37
HCV genotype		
1a	13	-
1b	43	-
2	1	-
3	2	-
Not determined	1	-

HCV, hepatitis C virus; SD, standard deviation; ALT, alanine transaminase; AST, aspartate aminotransferase.

Expression and purification of HCV F protein. Cloning of fragments was performed as described previously (17). Briefly, the fragment of F protein corresponding to residues 2-144 of the HCV 1b ORF was obtained using PCR. The purified F protein PCR products were then cloned into the expression vector pColdII (Takara Bio Inc., Shiga, Japan), upstream of a hexahistidine tail, allowing overexpression in *E. coli* strain BL21 (DE3) with 1 mmol/ml isopropyl-β-D-thiogalac topyranoside at 25˚C for 6 h. Subsequently, the precipitated bacteria were centrifuged and resuspended in binding buffer [20 mM imidazole, 8 M urea, 0.01 M phosphate-buffered saline (PBS), pH 7.4]. Following sonication on ice, soluble fractions were purified under denaturing conditions on Ni Sepharose High-Performance affinity media (GE Healthcare, Waukesha, WI, USA) with 500 mM imidazole. Analysis of the eluted fraction was performed using Coomassie Blue staining following 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The recombined F protein was subjected to SDS-PAGE and western blot analysis as described previously using an anti-histidine-tag antibody (Sigma, St. Louis, MO, USA) and patient sera at dilutions of 1:1,000 and 1:100, respectively (24).

Detection of anti-F protein and -Core antibodies. The indirect ELISA was performed as described by Kong *et al* (17). Briefly, the microtiter plates were coated with 100 μl purified F protein (1.25 μg/ml) or Core (1.25 μg/ml, amino acid 2-192 of HCV 1b subtypes) (Abcam, Cambridge, MA, USA) in 50 mM sodium carbonate buffer (pH 9.6) and incubated overnight at 4˚C. Wells were blocked with 0.05% Tween-20 in phosphate-buffered saline (pH 7.4) containing 1% albumin bovine V (Roche, Basel, Switzerland) for 2 h. Following five washes, 100 μl diluted sera sample (1:100) was added to duplicate wells and incubated at 37˚C for 1 h. Subsequently, the wells were incubated with peroxidase-conjugated AffiniPure goat anti-human immunoglobulin G whole antibody (1:5,000, Sigma), at 37˚C for 30 min. The substrate reaction was then developed by adding 3,3',5,5'-tetramethylbenzidine buffer, and terminated after 10 min in the dark by the addition of 50 μl 2 M H_2SO_4. The absorbance was read at 450 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). An optical density value ≥2.1-fold that of the negative control was considered to be positive.

Separation of PBMCs or PDCs from peripheral blood. PBMCs were isolated from the peripheral blood of patients with chronic HCV and healthy donors by Ficoll-Hypaque density gradient centrifugation according to the manufacturer's instructions (TBD Biotech, Tianjin, China). PBMCs were washed and resuspended ($1x10^7$ cells/ml) in 96-well plates (Corning, New York, NY, USA) with PBMC culture medium [containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1% nonessential amino acids (Sigma), 50 U/ml ampicillin (Sigma) and 50 U/ml streptomycin (Sigma) in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA)] at 37˚C and in 5% CO_2.

PDCs were isolated from PBMCs using a Plasmacytoid Dendritic Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, the PBMCs ($1x10^8$ cells/100 μl) were incubated in separation buffer [containing 0.5% bovine serum albumin and 2 mM EDTA in 0.01 mM PBS], and centrifuged at 3,000 x g for 10 min. Cells were then resuspended ($1x10^8$ cells/400 μl) and added to 100 μl PDC Biotin-Antibody Cocktail (Miltenyi Biotec) for 10 min at 4˚C, prior to undergoing centrifugation and the addition of 400 μl separation buffer and 100 μl Anti-Biotin MicroBeads (Miltenyi Biotec) for 15 min at 4˚C. PBMCs were then washed using 10 ml buffer and filtered through a magnetic-activated cell sorting column (Miltenyi Biotec). Following staining with streptavidin-phycoerythrin and anti-CD123-allophycocyanin antibodies, the purity of the isolated PDCs was determined by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA, USA) analysis to be 90% (data not shown). PDCs included the following biomarkers: $CD123^+$, $CD11c^-$, $HLA\text{-}DR^+$ and $Lineage^-$ (CD3, CD14, CD16, CD19, CD20, CD56) (25). PDCs were cultivated in RPMI-1640 medium with 10% FBS, 50 U/ml ampicillin, 50 U/ml streptomycin and 50 ng/ml recombinant IL-3 (rIL-3; PeproTech, Rocky Hill, NJ, USA) at 37˚C and in 5% CO_2.

Analysis of cytokine production by PBMCs and PDCs using ELISA. To detect the production of IFN-α and IL-10 by PBMCs in response to HCV proteins, $1x10^7$ cells/ml PBMCs were incubated in culture medium in the presence or absence of 5 mM unmethylated CpG oligonucleotide sequences of type A (CpG-A; InvivoGen, San Diego, CA, USA), 2.5 μg/ml Core, 2.5 μg/ml F protein and 10 μg/ml anti-IL-10 antibody (eBioscience, San Diego, CA, USA) for 48 h at 37˚C (26). Following incubation, the cell-free supernatant from the media was collected and IFN-α or IL-10 levels were measured using an ELISA kit (eBioscience). To exclude endotoxin contamination of the recombinant proteins, polymyxin B sulfate (Sigma) was added at 100 μg/ml to inhibit any endotoxin-induced cytokine production as described previously (27).

The level of IFN-α secreted by PDCs ($1x10^6$ cells/ml) was analyzed using an ELISA kit (eBioscience), following stimulation with 5 mM CpG-A, 2.5 μg/ml Core and 2.5 μg/ml F protein for 48 h at 37˚C. Polymyxin B sulfate (100 μg/ml) was also added to exclude endotoxin contamination (27).

Detection of PDC apoptosis by flow cytometry. PDCs were cultivated in RPMI-1640 medium with 10% FBS, 50 ng/ml rIL-3 (PeproTech), 50 U/ml ampicillin (Sigma) and 50 U/ml streptomycin (Sigma) in the presence or absence of 0.1 ng/ml rIL-10 (eBioscience) at 37˚C and in 5% CO_2 for 48 h. PDCs were then collected, washed twice with PBS and resuspended in 500 μl 1% paraformaldehyde in PBS. Subsequently, the harvested cells ($1x10^6$) were double-labeled with fluorescein isothiocyanate-Annexin V (BD Biosciences) and propidium iodide (BD Biosciences) according to the manufacturer's instructions (28). Unlabeled cells were used as a control to determine the threshold value of FL1 and FL2 channels. The rate of apoptosis was calculated using the percentage of apoptotic cells against the whole cell population.

All experiments were set up in triplicate, and the mean ± standard deviation (SD) was used in the statistical analysis.

Statistical analysis. The quantitative data are presented as the mean ± SD. Statistical analyses were performed using the Mann-Whitney U test or Kruskal-Wallis test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were conducted using SPSS statistical software 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Prevalence of specific anti-F or -Core antibodies in the sera of patients with chronic HCV and of healthy controls. To analyze the seroprevalence of the anti-F protein or -Core antibodies in patient sera, ELISA was performed using Core and purified recombinant F protein. In this study, all patients with chronic HCV were positive for anti-Core antibodies, and 65% (39/60) were positive for anti-F protein antibodies. By contrast, none of the healthy individuals exhibited detectable anti-Core or -F protein antibodies in the sera. The patients positive for the anti-F protein antibody included 30 patients of HCV 1b genotype, eight patients of HCV 2 genotype and one patient of HCV 3 genotype.

Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) correlate with anti-F protein antibody prevalence in patients with chronic HCV and healthy controls. As illustrated in Fig. 1, it was observed that the level

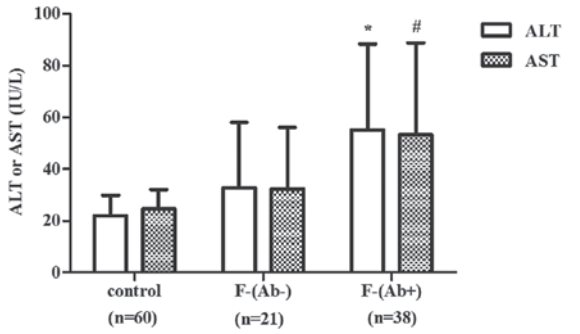

Figure 1. Level of ALT or AST in the sera of patients with chronic HCV and healthy controls. Values are presented as the mean ± standard deviation. *P=0.021, between the groups of F-(Ab$^-$) and F-(Ab$^+$) patients; #P=0.036, between the groups of F-(Ab$^-$) and F-(Ab$^+$) patients. Control, healthy control; F-(Ab$^-$), patients negative for anti-F protein antibody; F-(Ab$^+$), patients positive for anti-F protein antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus.

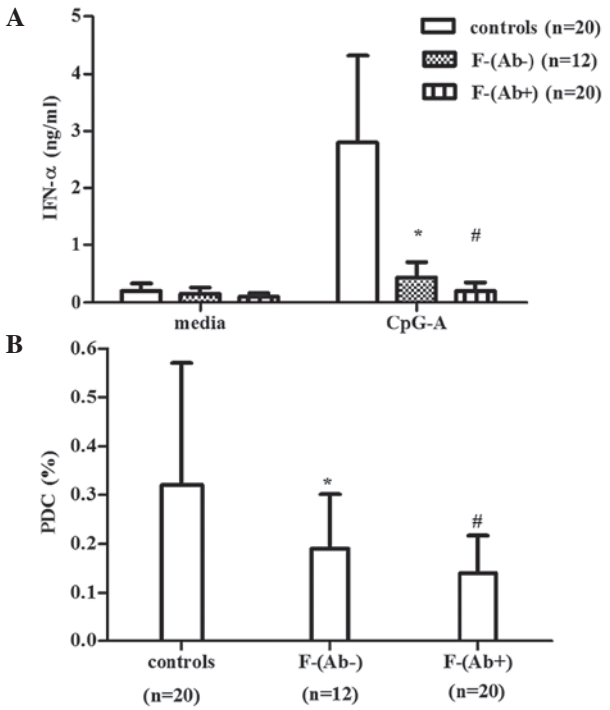

Figure 2. Patients with chronic HCV infection exhibit reduced IFN-α secretion by PBMCs and reduced frequency of PDCs. (A) IFN-α was secreted by PBMCs (1x10^7 cells/ml) and detected using ELISA in the absence or presence of CpG-A for 48 h *in vitro*. Samples from patients positive for anti-F protein antibody, patients negative for anti-F protein antibody and healthy controls were assessed. (B) The frequency of PDCs in patients positive for anti-F protein antibody, patients negative for anti-F protein antibody and healthy individuals was analyzed using flow cytometry. *P<0.05, between the groups of F-(Ab$^-$) and control; #P<0.05, between the groups of F-(Ab$^+$) and F-(Ab$^-$). Values are presented as the mean ± standard deviation. Controls, healthy control; F-(Ab$^-$), patients negative for anti-F protein antibody; F-(Ab$^+$), patients positive for anti-F protein antibody; HCV, hepatitis C virus; IFN, interferon; PBMCs, peripheral blood mononuclear cells; PDCs, plasmacytoid dendritic cells; CpG-A, CpG oligonucleotide sequences of type A.

of ALT in patients positive for the anti-F protein antibody was significantly higher than that of patients negative for the anti-F protein antibody (P=0.021). A significant difference was also identified in AST levels between patients positive for the anti-F protein antibody and those negative for the anti-F protein antibody (P=0.036). One patient with chronic HCV who was positive for the anti-F protein antibody was excluded due to a lack of ALT/AST data.

Patients with chronic HCV exhibit a reduced production of IFN-α by PBMCs and a lower frequency of PDCs. To analyze the cellular immune response against F protein in patients with chronic HCV, the level of IFN-α secreted by PBMCs in the presence or absence of anti-F protein antibody was assessed using ELISA. A significantly reduced level of IFN-α secretion was observed in patients that were positive for anti-F protein antibody, compared with those that were negative for anti-F protein antibody or the healthy controls (P<0.05) (Fig. 2A). Moreover, the level of IFN-α was lower in patients that were negative for the anti-F protein antibody than in the healthy controls (Fig. 2A). This study also assessed the frequency of circulating PDCs in patients with chronic HCV compared with healthy individuals (Fig. 2B). The results revealed that the frequency of PDCs in patients negative and positive for the anti-F protein antibody was 0.19 (±0.11) and 0.14 (±0.08), respectively, and 0.32 (±0.25) in the healthy controls. The difference in frequencies of circulating PDCs between patients positive- and negative- for the anti-F protein antibody was considered significant (P<0.05). Significance was also maintained when comparing patients negative for the anti-F protein antibody and the healthy controls (P<0.05).

HCV F protein and Core inhibit IFN-α production in PBMCs. Based on the results described above, it was hypothesized that, similar to Core, F protein may impair IFN-α production in PBMCs or PDCs from peripheral blood (22). As shown in Fig. 3A and B, the results revealed that F protein or Core inhibited the IFN-α production by PBMCs in patients with chronic HCV, compared with that of the healthy controls treated with Core or F protein combined with CpG-A. Consistent with a previous study (22), F protein or Core did not influence the secretion of IFN-α by PDCs isolated from PBMCs in healthy controls. This finding indicated that F protein may suppress the production of IFN-α by PBMCs or PDCs in patients with chronic HCV. In addition, the level of IL-10 produced by PBMCs in the presence of Core or F protein was analyzed. As expected, when combined with CpG-A, Core and F protein increased the secretion of IL-10 by PBMCs in healthy controls and patients with chronic HCV (Fig. 3C). Moreover, the level of IFN-α was increased following the neutralization of the IL-10 in the media and Core or F protein stimulation *in vitro* (Fig. 3D). In combination, these data indicated that F protein or Core may trigger the production of IL-10, which may influence the secretion of IFN-α by PBMCs or PDCs.

HCV F protein and Core induce PDC apoptosis in patients with chronic HCV and healthy controls. It was hypothesized that the reduced IFN-α production was likely to correlate with HCV protein-induced PDC apoptosis. As shown in Fig. 4, upon stimulation with HCV F protein or Core, a significant increase in PDC apoptosis was detected in patients with chronic HCV. In contrast to Core, HCV F protein did not increase PDC apoptosis in healthy controls, unlike that observed in the patients with chronic HCV. Moreover, it was observed that the rate of

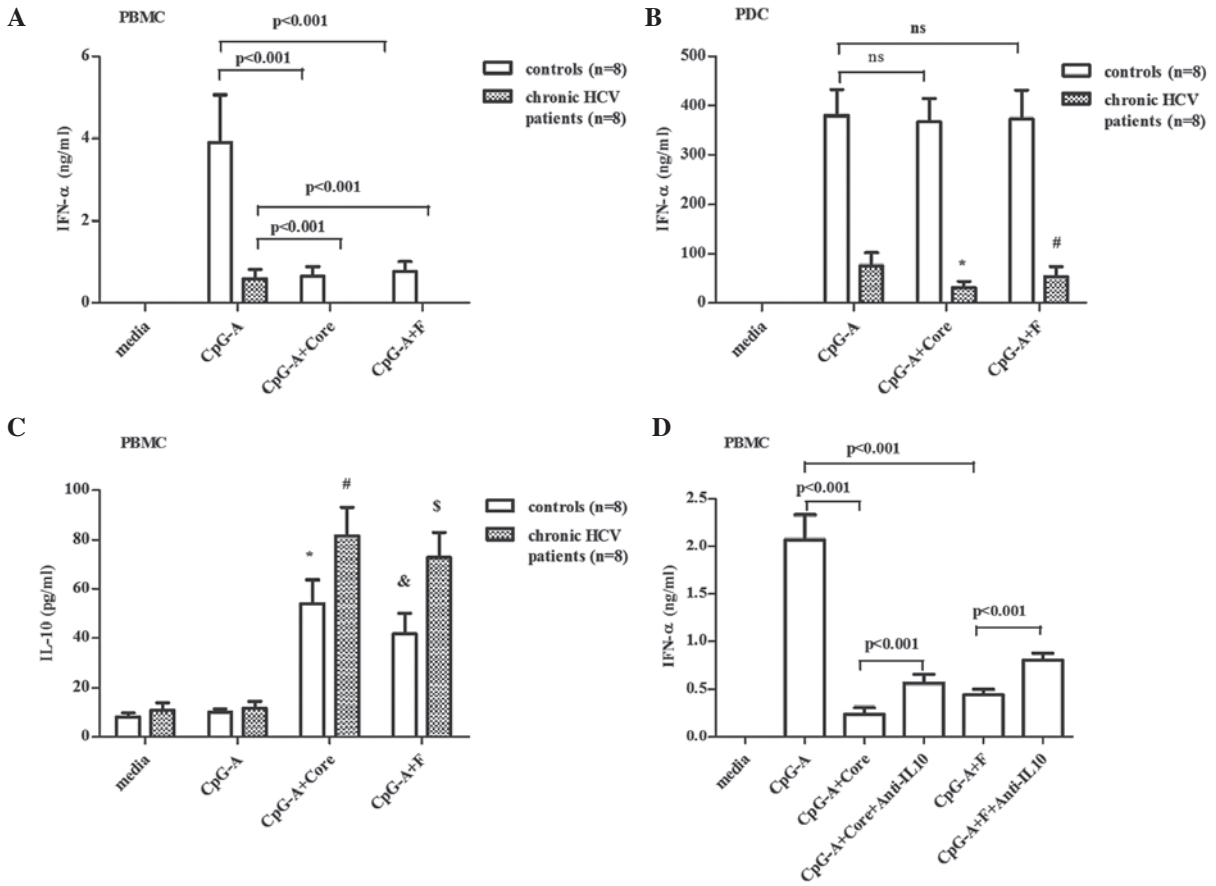

Figure 3. Analysis of IFN-α or IL-10 production in PBMCs or PDCs from patients with chronic HCV and healthy controls. (A) IFN-α secretion by PBMCs ($1x10^7$ cells/ml) from patients with chronic HCV and healthy controls, stimulated with or without Core or F protein, was measured using ELISA. (B) IFN-α secretion by isolated PDCs ($1x10^6$ cells/ml), stimulated as described above, was measured using ELISA. *P<0.001, between the CpG-A and CpG-A+Core groups; #P<0.001, between the CpG-A and CpG-A+F groups; ns, not statistically significant difference. (C) IL-10 secretion by PBMCs stimulated as described above was detected using ELISA. *P<0.001, between the CpG-A and CpG-A+Core groups of healthy controls; #P<0.001, between the CpG-A and CpG-A+Core groups of patients with chronic HCV; &P<0.001, between the CpG-A and CpG-A+F groups of healthy controls; $P<0.001, between the CpG-A and CpG-A+F groups of patients with chronic HCV. (D) IFN-α was analyzed using ELISA following PBMC ($1x10^7$ cells/ml) stimulation with Core or F protein in combination with CpG-A, in the absence or presence of anti-IL-10 antibody. Values are presented as the mean ± standard deviation. Controls, healthy control; Core, HCV Core; F, HCV F protein; anti-IL-10, anti-IL-10 antibody; IFN, interferon; IL, interleukin; PBMCs, peripheral blood mononuclear cells; PDCs, plasmacytoid dendritic cells; HCV, hepatitis C virus; CpA-G, CpG oligonucleotide sequences of type A.

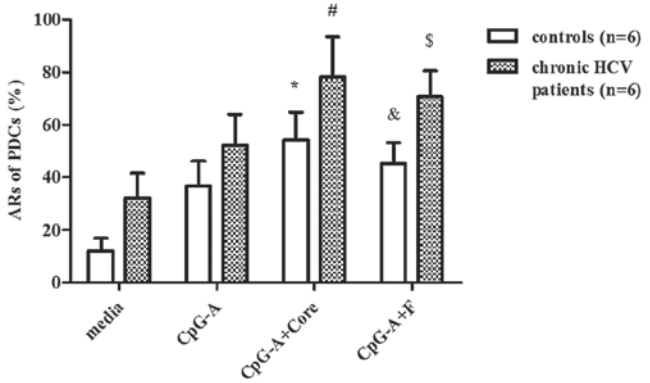

Figure 4. HCV F protein and Core induce PDC apoptosis in patients with chronic hepatitis and healthy controls. PDC apoptosis rates were analyzed using flow cytometry, following 48 h *in vitro* cultivation, with or without F protein or Core in combination with CpG-A. Values are presented as the mean ± standard deviation. *P=0.013, between CpG-A- and CpG-A+Core-stimulated healthy controls; #P=0.008, between CpG-A- and CpG-A+Core-stimulated patients with chronic HCV; &P=0.119, between CpG-A- and CpG-A+F-stimulated healthy controls; $P=0.015, between CpG-A- and CpG-A+F-stimulated patients with chonic HCV. HCV, hepatitis C virus; PDCs, plasmacytoid dendritic cells; AR, apoptosis rate; CpA-G, CpG oligonucleotide sequences of type A.

apoptosis in patients with chronic HCV was higher than in the group of healthy controls. As shown in Fig. 4, in the healthy controls, when stimulated with Core and F protein, the PDC apoptosis rates were 54.29 and 45.32%, respectively. In the patients with chronic HCV, when treated with Core or F protein, the PDC apoptosis rates were 78.17 and 70.63%, respectively.

Discussion

The clearance and persistence of an initial viral infection may be influenced by a number of factors associated with host, virus and environment. However, the specific mechanism of the immune response to HCV and the interaction between HCV and various host immune cells, remain unclear. HCV F protein is a derivative of the HCV core protein, the biological functions and pathogenicity of which have yet to be elucidated. In this study, it was found that the seroprevalence of anti-F protein antibodies was 65% in patients with chronic HCV, which was consistent with the study by Komurian-Pradel *et al* (29). Moreover, the study revealed that the anti-F protein antibodies were not limited to HCV 1b subtypes. It was also observed that

the levels of ALT/AST in patients positive for the anti-F protein antibody were significantly higher than those in patients negative for the anti-F protein antibody. This indicates that an association may exist between F protein and ALT/AST levels in patients with chronic HCV.

IFN-α is predominantly produced by PDCs, and provides a bridge between the innate and adaptive immune responses (30,31). Dolganiuc *et al* (22) reported that HCV Core triggered IL-10 and tumor necrosis factor (TNF)-α production, induced PDC apoptosis and impaired PDC capacity to produce IFN-α. HCV NS5 is also capable of impairing PDC function and decreasing PDC levels in patients with chronic HCV infection (25). Consistent with previous studies, it was observed in the present study that, similar to Core, F protein decreased the production of IFN-α and the frequency of circulating PDCs in peripheral blood from patients with chronic HCV. Therefore, it was hypothesized that the reduction in IFN-α levels may be associated with the low frequency of PDCs observed in patients with chronic HCV infection. To test this hypothesis, the production of IFN-α by PBMCs and PDCs treated with F protein or Core was analyzed *in vitro*. It was shown that the HCV F protein, like Core, inhibited the production of IFN-α by PBMCs from patients with chronic HCV and healthy controls. In addition, a similar reduction in IFN-α secretion by PDCs was observed in patients with chronic HCV, but not in the group of healthy controls. In our opinion, the cellular or humoral microenvironment is involved in the function of PDCs; it is possible that the PDC function of the patients has been interfered with by a virus or virus protein, so the protein expression and secretion may not be the same as that of healthy controls. It has been reported that HCV NS5 is capable of impairing PDC function (25). IFN-α has been indicated to have a role in the inhibition of viral replication, the activation of natural killer (NK) cell cytotoxicity and B cell antibody generation. Therefore, it was hypothesized that the reduced IFN-α production was likely to lead to insufficient cytotoxic T lymphocyte (CTL) response, or poor neutralizing capacity of antibodies (31). This suggested that F protein, like Core, may be involved in suppressing the cellular immune response.

Cytokines in the host microenvironment have an important role in the immune response against pathogenic microorganisms. For example, HCV Core interaction with IL-10 reduces IFN-α production in PBMCs or PDCs, and has been considered to be one of the main factors leading to chronic hepatitis (22,32,33). In this study, it was demonstrated that the production of IL-10 by PBMCs increased upon stimulation by Core or F protein in patients with chronic HCV and healthy controls. This finding is consistent with those by Dolganiuc *et al* (22) who found that Core induced IL-10 production in PBMCs. Notably, the present study demonstrated that antibody neutralization of IL-10 restored the ability of PDCs to produce IFN-α following stimulation with Core or F protein. These data indicated that the IL-10 production induced by Core or F protein impaired the capacity of PDCs to produce IFN-α. It is well-established that IL-10, a type 2 T helper (Th2) cell cytokine, is an immunomodulatory cytokine that has a crucial role in inhibiting immune and inflammatory responses by blocking antigen-presenting capacities of monocytes/macrophages (34). Moreover, levels of IL-10 alone were shown to be sufficient to dictate lymphocytic choriomeningitis virus (LCMV) clearance or persistence in an LCMV model of chronic viral infection (18). Martin-Blondel *et al* (35) also reported that the low levels of IL-10 production may be beneficial in establishing an effective immune response and spontaneous HCV clearance. Furthermore, the increased IL-10 production by PBMCs may promote naive T (Th0) cells to differentiate into Th2 cells, leading to a Th2-dominant profile (IL-4, IL-10). The Th2 profile, unlike the type 1 T helper (Th1) cell profile, may contribute to the development of chronicity. Based on these findings, it was hypothesized in the present study that the downregulation of IFN-α production and upregulation of IL-10 production by PBMCs may disturb the balance of Th1/Th2 cytokines and lead to disease development. Furthermore, PDCs are the predominant source of IFN-α production in response to viral infection, the most potent type of antigen-presenting cell in the host and are involved in the regulation of innate and adaptive immune responses (36-38). Degeneration of PDC function in patients with chronic HCV may be attributed to multiple factors, including cellular infection, necrosis, apoptosis or cytokine-mediated dysfunctional regulation. The present study assessed PDC apoptosis using flow cytometry and revealed that the F protein, like Core, induced apoptosis in PDCs in patients with chronic HCV; however, these proteins had little effect on the group of healthy controls. In combination, these data indicate that the reduced IFN-α production may account for the induction of PDC apoptosis and upregulation of IL-10 induced by HCV F protein. PDCs are the main source of IFN-α. Upon viral infection, they produce large amounts of IFN-α, which block viral replication and stimulate innate and adaptive immune responses (30,31). However, the expression of F protein during HCV infection could reduce the IFN-α secretion. Therefore, we consider that the reduction in IFN-α induced by F protein may contribute to the reduced antiviral response of the host immune system during HCV infection. In addition, Lozach *et al* (39) demonstrated that the HCV envelope glycoprotein E2, like the human immunodeficiency virus envelope glycoprotein gp120, bound the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and the liver endothelial cell lectin L-SIGN through high-mannose N-glycans. The interaction of the virus with DC-SIGN had a role in viral dendritic cell (DC) entry, and may also be detrimental for DC interaction with T cells during antigen presentation (40). Therefore, it was hypothesized that the increased apoptosis of PDCs induced by HCV Core or F protein may be a response to HCV infection and low virus dissemination, consistent with findings by Siavoshian *et al* (33) that HCV proteins are associated with modulation of apoptosis pathways in mature DCs.

In conclusion, this study indicated that the low production of IFN-α in patients with chronic HCV may be attributed to the low frequency of PDCs in the peripheral blood, PDC apoptosis and F protein-induced PDC dysfunction. Moreover, it was suggested that the F protein, unlike Core, has a unique role in the pathogenesis of chronic hepatitis. It was proposed that the F protein-induced imbalance in IFN-α and IL-10 production decreased the protective immune response, and promoted a cytokine imbalance in favor of

immunosuppression. Furthermore, it was suggested that the F protein, expressed during natural HCV infection induced apoptosis in PDCs and reduced the likelihood of the transmission of the virus to neighboring hepatocytes or T cells during antigen presentation. This study may offer an insight into the potential for therapeutically targeting F protein in patients infected with HCV; however, there remains a requirement for further research regarding F protein biological function and pathogenesis.

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