Th1 and Th2 cytokines are elevated in HCV-infected SVR(−) patients treated with interferon-α

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ABSTRACT

Interferon-α-based treatment is a standard therapy to cure hepatitis C virus-infected patients. However, the reasons for the failure of interferon-α treatment in some patients have not been fully elucidated. We evaluated the differences in the expression levels of various cytokines among patients with and without sustained viral response (SVR). We found that the chemokines (MIG and IP-10) and inflammation-related cytokines (IL-6) were transiently elevated in patients with SVR(+) before interferon-α treatment and in the early phase of treatment (week 2), indicating that these cytokines may be related to viral clearance. Furthermore, higher serum levels of Th1 and Th2 cytokines (IL-2, IL-4, IL-5, IL-10, tumor necrosis factor, and IFN-γ) were observed in SVR(−) than in SVR(+) patients, indicating that they may be associated with ineffective anti-HCV immune response. Our data revealed that the patterns of cytokines varied greatly between SVR(+) and SVR(−) patients before and after IFN-α treatment.

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Approximately 170 million people worldwide are infected with the hepatitis C virus (HCV), the causative agent of non-A, non-B hepatitis [1]. The infection becomes chronic in 50–80% of cases, which can lead to cirrhosis and hepatocellular carcinoma [2,3]. Despite the progressive understanding of the structure and pathological pathway of HCV, neither an effective vaccine nor effective therapy has been discovered. The current standard therapy for chronic hepatitis C is IFN-α combined with ribavirin; however, the rate of patients with poor response to the IFN-ribavirin combination therapy is high. According to previous reports, combination therapy is efficacious in clearing HCV infection in only 40% of patients [4,5]. The reason for non-responsiveness may be associated with the interaction between HCV and human immune response, leading to an increase in the rate of HCV quasispecies diversification [6,7].

In chronically infected individuals, HCV persists despite the presence of both humoral and cellular immune defenses [8,9]; yet little is known regarding the factors leading to viral clearance and persistence. In HCV-infected patients, the significance of the cytokine profile has been addressed in clinical studies. Treatment with IFN-α is accompanied by a decreased secretion of the T-helper 2 (Th2) cytokines, i.e. interleukin-4 (IL-4) and IL-10 [10], which proliferate, activate, and cause the B lymphocytes to produce antibodies. Considering the upstream effectors, IFN-α—which is important in T cell development—stimulates the production of Th1 but restrains Th2 cells in cell line studies [11,12]. This suggests that Th1 rather than Th2-type cytokines may be the profitable effecters of IFN-α in eliminating the HCV infection.

Previous studies have focused on the manner in which Th1/Th2 cytokines behave in HCV viral clearance and persistence [12–18]. The aim of the present study is to investigate how a broader range of cytokines behaves in HCV clearance, including inflammation-related cytokines and chemokines. To achieve this, we chose cytometric bead array analysis to simultaneously identify in serum dynamic profiles of 14 types of cytokines known to be associated with HCV clearance during IFN-ribavirin combination therapy.

Materials and methods

Patients and blood samples. Patients who had positive anti-HCV, detectable serum HCV RNA (real-time PCR assay with LightCycler RNA Amplification Kit SYBR Green I; Roche Diagnostics, Branchburg,

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NJ, USA), and elevated serum alanine aminotransferase (ALT) levels were routinely evaluated for antiviral therapy at our hepatology unit. Peginterferon alpha 2a was given as a fixed dose of 180 μg/week and peginterferon alpha 2b was given as 1.5 μg/kg/week. Genotype analysis of HCV was performed with a real-time quantitative PCR assay followed by melting curve analysis. We routinely measured serum albumin, aspartate aminotransferase (AST), ALT, total bilirubin, creatinine, fasting sugar, triglyceride, hemoglobin, white blood cells (WBC), and platelets before treatment, and monitored those factors monthly during the treatment and post-treatment follow-up periods. The serum HCV RNA levels were determined before treatment and at 4, 12, and 24 weeks (indicated as TW0–24) during treatment and at 24 weeks after treatment (TW48). Early virological response (EVR) was defined as the undetectable serum HCV RNA or a minimum of a 2-log decrease in serum HCV RNA from the baseline level after 12 weeks of therapy. Sustained virological response (SVR) was defined as the undetectable serum HCV RNA 24 weeks after the end of therapy. Blood samples were collected for the hematological, biochemical, and virological analyses. Twenty-eight patients whose stored plasma samples were available were enrolled into the current study. 17 out of the 28 patients were EVR(+) and SVR(+) and the other 11 patients were EVR(+) but SVR(−). Nine patients in the EVR(+)/SVR(+) group and 8 patients in the EVR(+)/SVR(−) group were of genotype 1, and the remaining patients were of genotype 2 (Table 1).

Reagents. Various Cytometric Bead Array (CBA) Cytokine Kits (BD), including the Human Inflammation Kit, the Human Chemokine Kit and the Human Th1/Th2 Cytokine Kit were used to quantitatively measure the cytokine profiles using flow cytometry. These kits measure a wide-range of cytokines, such as interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70), tumor necrosis factor (TNF), interferons (IFN-γ), and chemokines (IL-8, IFN-γ-induced protein 10; IP10, RANTES, monokine-induced by IFN-γ; MIG, and monocyte chemoattractant protein-1; MCP-1).

Detection of cytokine profile. An Aliquot of each human cytokine capture bead suspension was briefly vortexed and the supernatant was removed. The bead pellet was resuspended in the serum enhancement buffer, and vortexed briefly. After a 30-min incubation, protected from light, an aliquot of mixed beads was transferred to each assay tube. In the meantime, cytokine standards were reconstituted and subsequently serially diluted. The serum samples were diluted by the desired dilution factor to ensure that their mean fluorescence values fell within the standard curve. Afterwards, 50 μl of PE detection reagent and 50 μl of standard dilutions or test samples were added to the appropriate sample tubes. After 3 h of incubation, the samples were washed with wash buffer and centrifuged. The supernatant was carefully aspirated and discarded; subsequently, 300 μl of wash buffer was added to each assay tube. Samples were then analyzed on a flow cytometer.

BD FACSComp software was used to set up the flow cytometer. Data were acquired on BD CellQuest Software and formatted for subsequent analysis using BD CBA software.

Statistical analysis. Results were analyzed by nonparametric tests using SPSS (version 9.0; SPSS Inc., Chicago, IL,USA) and GraphPad Prism (version 5.0; GraphPad Software Inc., San Diego, CA, USA). The difference in the expression levels of cytokines between SVR(+) and SVR(−) patients was evaluated using the Mann–Whitney U test. The variation of specific cytokines measured at different time courses was analyzed by the Kruskal–Wallis test for multiple groups. Multiple comparisons were calculated by the Dunn's test, which is equivalent to parametric post hoc multiple comparison tests such as the Tukey's test. A probability value of less than 0.05 was considered statistically significant.

Results

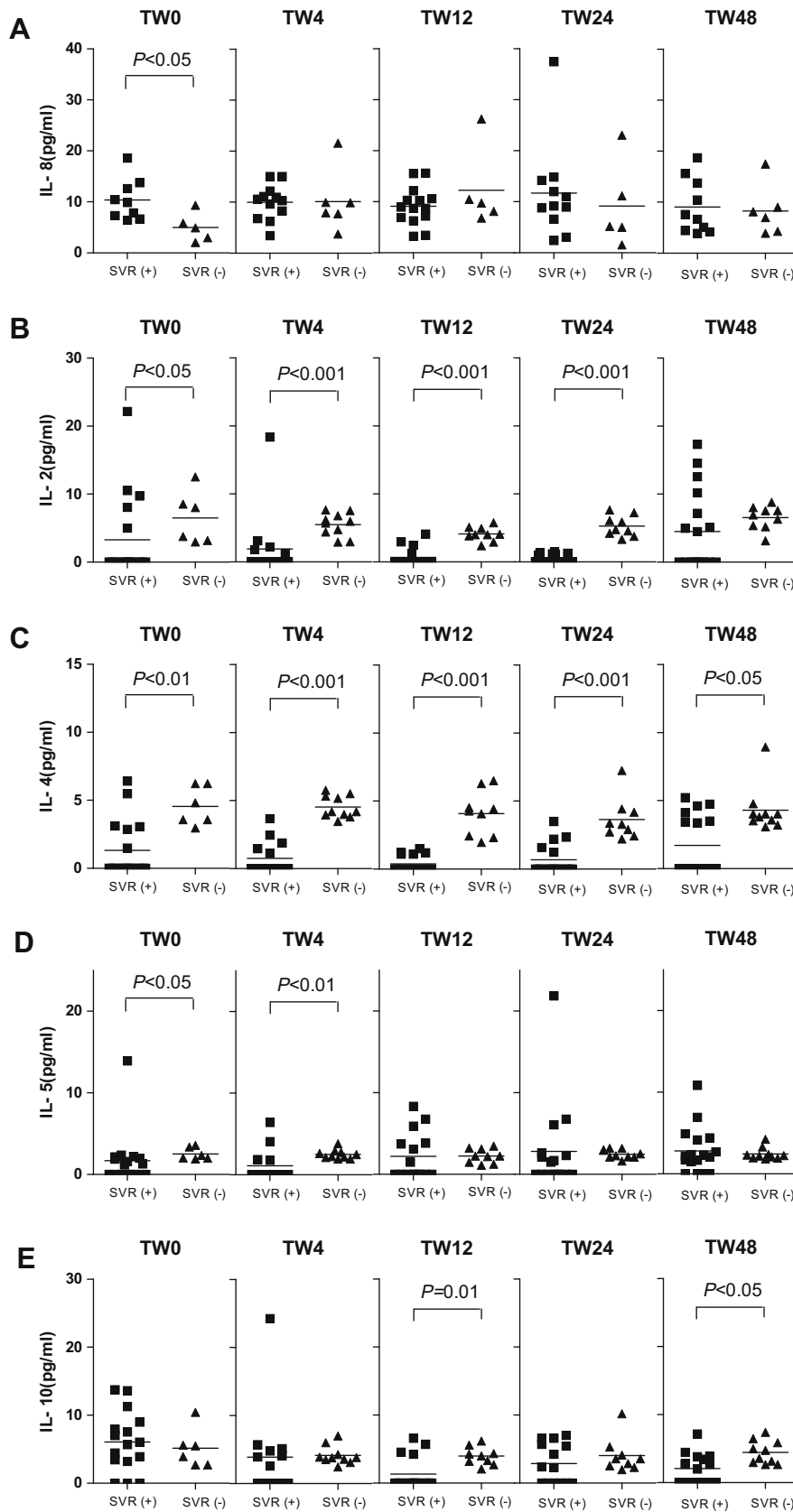### Cytokines expression levels were changed during IFN-ribavirin treatment

The various parameters between these 2 groups (17 EVR(+)SVR(+) and 11 EVR(+)SVR(−) patients) were not significantly different before treatment (ages: $P = 0.08$; viral load: $P = 0.13$; ALT: $P = 0.6$ and AST: $P = 0.2$). The treatment time course was defined as before treatment (TW0), early phase of treatment (weeks 2–4, TW2–4), late phase of treatment (weeks 12–24, TW12–24), and after treatment (week 48, TW48). The levels of various cytokines changed in the EVR(+)SVR(+) patients as the treatment period progressed. MIG and IP-10 were expressed at higher levels before treatment than during the early- and late-phases, and also after treatment (Table 1, Supplementary data 1A and B). However, IL-6, which emerged during the acute-phase response of inflammation as an endogenous pyrogens, was elevated in the early phase (TW2) (Table 1, Supplementary data 1C). The expression levels of MIG, IP-10, and IL-6 were elevated before and during the early phase of treatment, but decreased after IFN-α treatment. On the contrary, IL-2 and IL-4 were not elevated until TW48. (Table 1, Supplementary data 1D and E). IFN-γ was unchanged throughout TW0–TW24 but was significantly elevated after treatment (Table 1, Supplementary data 1F). The pattern of other cytokines in the serum samples collected from EVR(+)SVR(+) patients did not differ significantly within the entire period of treatment. Furthermore, all cytokine expression levels detected from 11 EVR(+)SVR(−) patients were unchanged from TW0 to TW48 (data not shown).

IL-8, a proinflammatory chemokine, was expressed at a higher level before treatment in SVR(+) than that in SVR(−) patients

The cytokine profiles of SVR(+) and SVR(−) patients were compared in order to elucidate whether there was correlation between viral clearance and cytokine patterns. As shown in Fig. 1A, IL-8 levels differed significantly between SVR(+) and SVR(−) patients before treatment (TW0); however, there was no significant difference between the groups after TW0.

Chemokines were expressed similar levels between SVR(+) and SVR(−) patients

The chemokines expression profiles were shown in Supplementary data 2. The expression levels of RANTES, MIG, MCP-1, and IP-10 in SVR(+) and SVR(−) patients were compared; however, no significant differences were found. The chemokine levels in HCV patients were all similar, whether the IFN treatment was successful or not.

Table 1
Clinical characteristics of SVR(+) and SVR(−) patients.

	Demographics			
	SVR(+)		SVR(−)	
Gender (female/male)	5/12		4/7	
Age, years (mean ± SD)	55.88 ± 9.02		49.09 ± 10.96	
Viral genotype (1a/1b/2a/2b)	3/6/6/2		0/8/3/0	
	Response levels			
	Before treatment	TW48	Before treatment	TW48
Serum HCV RNA (copies ± SD)	1.48 × 106/ml ± 2.95	<1000/ml	4.19 × 106/ml ± 5.21	6.26 × 105/ml ± 9.81
ALT (mean ± SD)	155.8 ± 77.8	26.7 ± 19.6	39.2l ± 102.9	49.1 ± 49.2
AST (mean ± SD)	102.7 ± 49.4	29.4 ± 19.5	78.9 ± 48.1	38.1 ± 23.1

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; SVR, sustained viral response.

Fig. 1. Differences of specific cytokines between SVR(+) and SVR(−) patients within 48 weeks. (A) IL-8; (B) IL-2; (C) IL-4; (D) IL-5; (E) IL-10; (F) TNF; and (G) IFN-γ. Horizontal bars indicated the mean values. The P value for statistical significance was indicated in the diagram. The significance of data was calculated using Mann–Whitney U test.
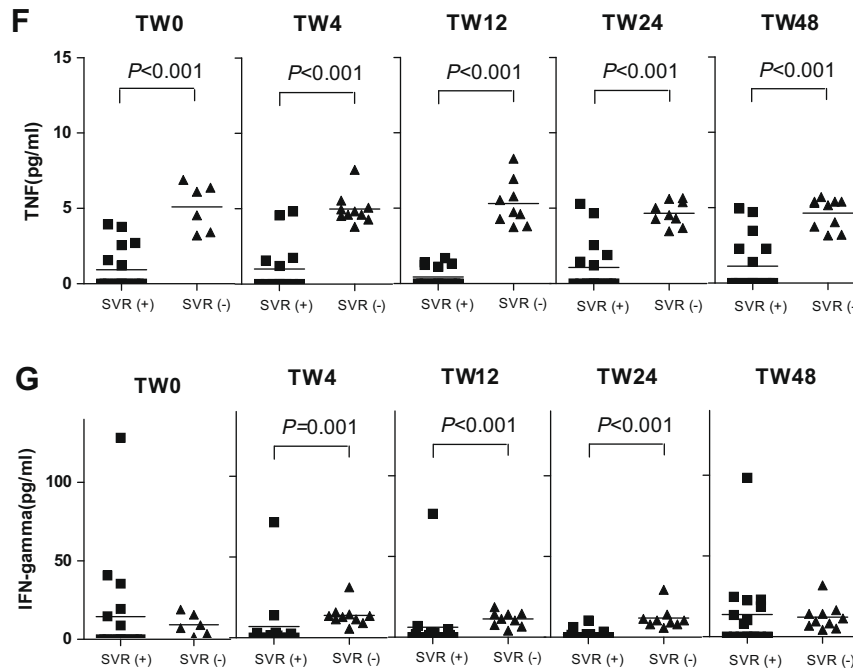
Fig. 1. (*continued*)

Both Th1 and Th2 cytokines were expressed at higher levels in SVR(−) patients

Before treatment, the amount of IL-2 in the serum of SVR(+) patients was lower than that in the serum of SVR(−) patients; this reduced level persisted through the late phase of treatment. There was no difference in the IL-2 levels between SVR(+) and SVR(−) patients at TW48 (Fig. 1B).

The amount of IL-4 was lower in SVR(+) patients than in SVR(−) at TW0, and significantly lower from TW4 to TW24. The difference at TW48 remained significant but not as marked as during TW4 through TW24 (*P* < 0.05) (Fig. 1C). IL-5 levels were lower in the initial state in SVR(+) patients than in SVR (−) patients, and the difference was significant in the early phase. However, the differences were statistically insignificant in the later phases (Fig. 1D). IL-10 was lower in SVR(+) than in SVR(−) patients until the late phase; this reduced level of IL-10 expression in SVR(+) patients was noted after treatment as well (Fig. 1E).

TNF was expressed at significantly lower levels in SVR(+) serum than in SVR(−) serum before, during and after treatment (*P* < 0.001) (Fig. 1F). The pattern of IFN-γ expression before treatment was similar in SVR(+) and SVR(−) patients; IFN-γ was expressed at significantly lower levels in SVR(+) patients than in SVR(−) during the early- and late-phases, but returned to the same level after treatment (Fig. 1G). Levels of the other cytokines were also compared; however, the amounts of the remaining cytokines were statistically indistinguishable between the 2 groups.

The characteristics of the quantitative cytokine expression levels between SRV(+) and SRV(−) are summarized in Table 2.

Discussion

In this study, a larger number of cytokines was investigated simultaneously in HCV-infected patients treated with IFN-α based therapy, who either achieved viral clearance or did not, to find out the causal relation between cytokine expression levels and HCV viral clearance. Differences in cytokine expression noted during the course of treatment may indicate an interaction between immune response and viral activity.

Acute hepatitis C progresses to chronic HCV infection in the majority of cases; therefore, it is important to understand the factors that result in chronic inflammation. IL-8, a proinflammatory chemokine, may inhibit the antiviral activity of IFN-α [19] and positively correlate with the stage of hepatic fibrosis and portal inflammation during HCV infection [20]. In the present study, IL-8 was found to be expressed at a higher level before treatment in SVR(+) than SVR(−) patients, which is in contrast with the findings of Polyak et al. [19]. However, IL-8 was also observed to have opposing antiviral and proviral effects depending upon the level of HCV replication and whether the infection was acute or chronic [21]. IL-8 may have antiviral effects against viruses with low replicative capacity; therefore, the proinflammatory status may have been inhibitory to HCV in the SVR(+) patients included in this study. Shapiro et al. reported that mRNA levels of IL-6 were

Table 2

Summarization of the differences noted in the cytokine expression levels within SVR(+) or between SVR(+) and SVR(−) patients.

Cytokine	Peak expression level in SVR(+)[*]	Expression level compared between SVR(+) and SVR(−)[#]
IL-2	TW48	SVR(−) > SVR(+) (TW0,4,12,24)
IL-4	TW48	SVR(−) > SVR(+) (TW0,4,12,24,48)
IL-5	N.D.	SVR(−) > SVR(+) (TW0,4)
IL-10	N.D.	SVR(−) > SVR(+) (TW12,48)
IL-8	N.D.	SVR(+) > SVR(−) (TW0)
IL-1β	N.D.	N.D.
IL-6	TW2	N.D.
IL-12p70	N.D.	N.D.
TNF	N.D.	SVR(−) > SVR(+) (TW0,4,12,24,48)
IFN-γ	TW48	SVR(−) > SVR(+) (TW4,12,24)
RANTES	N.D.	N.D.
MIG	TW0	N.D.
MCP-1	N.D.	N.D.
IP-10	TW0	N.D.

N.D., No difference.

[*] Time point of the highest expression level of the indicated cytokine.

[#] Summary of the expression levels of cytokines compared within the SVR(+) and SVR(−) patients at different time points. Time marked within the parentheses indicated period of significant difference.

elevated in SVR(+) patients after IFN-α treatment [17] Our data reveal that IL-6 was elevated in SVR(+) patients at TW2 (Table 1, Supplementary data 1C). Because of the early emergence of the differences in IL-8 and IL-6, the original levels of inflammation-related cytokines may be the leading factor driving the antiviral process.

IL-12 is a potent modulator of natural killer (NK) and T-cell function [22]. The production of IL-12 is critical for induction of Th1 immunity and is directed towards the elimination of intracellular pathogens and viruses. However, we found no difference in IL-12p70 between SVR(+) and SVR(−) patients (Table 2). Therefore, IL-12p70 alone may not be involved in viral persistence.

Gene expression levels of chemokine were investigated in chimpanzees with acute and chronic HCV infection [23,24] by gene microarray. In these reports, IFN-inducible protein 10 (IP-10) was observed that have increased in all infected animals. IP-10 is expressed by the liver in response to IFN and is an important chemokine for the recruitment of activated T and NK cells to the liver. MIG is another chemokine that acts as a chemoattractant for activated T cells [25]. However, the MIG expression was only mildly activated in some of the infected animals [23]. Further, levels of RANTES exhibited a decreasing trend but were not above the cutoff [23]. According to the present data, serum levels of MIG and IP-10 in SVR(+) patients significantly decreased after the initiation of IFN therapy (Table 1, Supplementary data 1A and B), whereas the levels of RANTES remained identical throughout the treatment. The pattern of the chemokine expression levels may indicate that the original levels of MIG and IP-10 are related to the rate of viral clearance.

The CTL- and Th1 cell-mediated [26] immune response seems to be the main factor that protects against HCV infection. In the chimpanzee model, test animals who cleared HCV generated strong CTL but poor antibody responses [27]. It has been suggested that the differentiation of activated T cells towards Th1 cells results in an improvement of hepatitis symptoms [28]. Previous studies have demonstrated various serum Th1 cytokine profiles in patients with chronic HCV infection. Although some reports have revealed low levels of IFN-γ in patients with HCV infection [16] or no increase in the levels of Th1 cytokines [17], other studies have reported that serum levels of Th1 cytokines, including those of IFN-γ and IL-2 were elevated in HCV-infected patients [10,15]. According to our data, the expression levels of Th1 cytokines (IL-2, IFN-γ, and TNF-α) were markedly increased in serum of SVR(−) patients before and after IFN-α treatment (Fig. 1B,1F, and 1G). The increases in the levels of IL-2 and TNF-α during and after treatment may indicate that these cytokines may be either associated with an ineffective antiviral response or exhausted any further Th1 response to IFN therapy in SVR(−) patients.

Robust humoral responses do not generally correlate with HCV viral clearance; furthermore, HCV-specific antibodies in acute response are often transient and disappear [29]. These trends suggest that a Th1-type rather than a Th2-type response may be beneficial for controlling and clearing HCV. Tsai et al. reported that the levels of IL-4 and IL-10 were elevated in chronic hepatitis C patients [30]. Rehermann et al. reported that patients who develop a chronic infection show a predominant Th2 response that down-regulates the Th1 response [2]. Our findings show that Th2 cytokines (IL-4, IL-5, and IL-10) were markedly increased in SVR(−) patients (Fig. 1C–E). The overall increases in Th2, which down-regulated the cell-mediated immune response may have led to viral resistance, even though high amounts of Th1 cytokine were expressed in serum.

Th1 and Th2 activation may result from the interference of the highly conserved HCV core protein and is known to be dependent upon the nuclear factor of activated T cells (NFAT) [31]. The genes of Th1/Th2 cytokines (IL-2, IL-4, TNF-α, IFN-γ, and granulocyte-

macrophage colony-stimulating factor) have binding sites for NFAT in their promoter regions [32]. This may explain the overall activation of Th cytokines in our study.

Because the ratio of SVR(+) patients to SVR(−) patients in the genotype 1 HCV-infected patients (9/8) seems to be lower than that in the genotype 2 HCV-infected patients (8/3), there seems to be genotype specific differences of the cytokine profiles between SVR(+) and SVR(−) patients. Therefore, the statistic significances of the cytokine levels between SVR(+) and SVR(−) patients infected by specific virus were calculated separately (Supplementary data 3). Seven cytokines mentioned above were found that expressed different levels between SVR(+) and SVR(−) patient. Interestingly, when calculating the significance in terms of genotype 1 or genotype 2 HCV-infected patients, some genotype specific differences were shown. The expression levels of IL-2, IL-5, IL-10, and IFN-γ were shown statistically significant between SVR(+) and SVR(−) patients who infected by genotype 1 HCV. However, these cytokines expression levels were found no significant difference between SVR(+) and SVR(−) patients, infected by genotype 2 HCV. It may indicate that the different genotypes HCV maybe respond to IFN-based therapy or resist it duo to the different cytokines profiles.

In summary, our data revealed that the patterns of cytokines varied greatly between SVR(+) and SVR(−) patients before and after IFN-α treatment. We found that cytokines IL-6 was transiently higher in SVR(+) patients in the early phase of IFN treatment. This indicates that inflammation in the early stages may be related to the viral clearance. Furthermore, IL-8 was observed to be higher in SVR(+) patients than in SVR(−) patients in TW0; moreover, the levels of the chemokines (MIG, and IP-10) in SVR(+) patients would significantly decreased following the initiation of IFN therapy starting suggesting that the levels of these chemokines should be measured prior to IFN treatment as indices of therapeutic efficacy. Finally, higher level of Th1 and Th2 cytokines (IL-2, TNF, IFN-γ, IL-4, IL-5, and IL-10) were higher in SVR(−) patients and this phenomenon of Th1/Th2 imbalance may be associated with ineffective anti-HCV immune response.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.114.

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