Rev Soc Bras Med Trop 51(6):731-736, Nov-Dec, 2018
doi: 10.1590/0037-8682-0137-2018

Major Article

Influence of the inflammatory response on treatment of hepatitis C with triple therapy

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Abstract

Introduction: Chronic hepatitis C is a leading cause of liver disease. Infection triggers an immediate immune response in the host that is mediated by humoral/cellular mechanisms. T cells respond to infection via secretion of cytokines, which inhibit or stimulate one another, leading to cytokine imbalance and ultimately affecting treatment. Studies using interferon (IFN) and ribavirin (RBV) showed that TCD8+ cells and cytokine levels are associated with sustainable virological response (SVR). However, studies that investigated the effects of triple therapy (TT) are limited. **Methods:** The study included hepatitis C virus (HCV)+ RNA, *naives*, genotype 1, ≥18 years, and advanced fibrosis (F≥3) patients. Samples were collected at baseline and after 12 weeks (W12) of TT. Six cytokines were analyzed by flow cytometry. **Results:** Of 31 patients, four were excluded (two deaths, one interrupted TT, and one F2 patient). Of the 27 remaining patients, 21 (78%) were cirrhotic. SVR was achieved in 63% of the patients. The patients had a mean age of 55.11 ± 10.03 years. Analyses at baseline showed that the chemokine CCL5/Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) (p=0.04) and interleukin (IL)-6 (p=0.02), which was associated with SVR. RANTES (p=0.04) and IL-8 (p=0.01) levels were associated with SVR at W12. **Conclusions**: Similar to patterns observed during double therapy, IL-6, IL-8, and RANTES levels were associated with SVR in TT, indicating the potential role of interferon in immune response to hepatitis C virus.

Keywords: Hepatitis C. Cytokine. Treatment.

INTRODUCTION

A total of 184 million people, corresponding to 3% of the world population, are estimated to be infected with the hepatitis C virus[1]. Infection with hepatitis C virus is considered to be one of the major causes of liver disease and leads to the development of cirrhosis and hepatocellular carcinoma[2].

Dual therapy with petylated interferon (PegIFN) and rivabirin was considered the standard treatment for chronic hepatitis C until the middle of 2011. However, low SVR rates were observed in patients with genotype 1, advanced fibrosis, and cirrhosis[3-6]. SVR is characterized by the absence of HCV RNA (undetected viral load or <12 IU/mL) by polymerase chain reaction at 24 weeks after completion of PegIFN-based therapy[7].

In 2011, a new class of drugs became available that included the protease inhibitors (PIs) Boceprevir and Telaprevir, which were administered during TT in combination with PegIFN and RBV. Treatment with TT increased SVR rates but was not sufficiently effective, especially in patients with cirrhosis[8,9]. Other factors, including the IR, are associated with low SVR rates[10]. Several studies have attempted to further investigate the mechanisms underlying the above findings[11-15]; however, the results remained ambiguous and controversial.

Infection with the hepatitis C virus triggers an immediate immune response in the host, which involves humoral and cellular mechanisms[16].

The T cell response is mediated by the secretion of cytokines, which participate in a molecular cascade wherein the cytokines can stimulate or inhibit one another, eventually causing cytokine imbalance[17,18]. In turn, cytokine imbalance leads to sustained inflammation and the development of liver fibrosis[19,20].

One study indicated that the poor response of CD4 and CD8 T cells observed during chronic infection could be attributed to immunological tolerance in the liver[21].

Several studies showed an association between the abundance of TCD8+ cells and SVR[14,22], and the quality of the

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Received 11 April 2018
Accepted 13 September 2018

T helper (Th)1 response in the baseline period, and the increase in Th2 cytokines during treatment may define treatment success or failure[11,14,23,24]. These studies were performed on patients treated with dual therapy (IFN alpha or PegIFN and RBV). Regardless of the treatment response of the patient, the levels of certain cytokines and chemokines were found to be upregulated in response to stimulation by IFN. However, IFN treatment could significantly alter the host response to treatment.

The present study aimed to evaluate whether the RVS is influenced by the IR by analyzing chemokine levels in patients undergoing TT treatment.

METHODS

The prospective study was conducted at the Botucatu Medical School (FMB/UNESP) from September 1, 2014 to July 30, 2016. The study was approved by the Research Ethics Committee of the hospital.

The inclusion criteria were as follows: *naive*, genotype 1, fibrosis 3 or 4 (METAVIR score system[25]) patients, male and female genders, ≥18 years old, candidates for TT (PIs: Boceprevir or Telaprevir), and treatment completion. Patients with hepatitis B and/or human immunodeficiency virus (HIV) co-infection, pregnant women, and users of illicit drugs and alcohol were excluded.

Samples were collected at two time points (baseline and W12 of treatment). Patients were stratified into two groups, namely, the G1 and G2 groups, according to their virologic response (G1: SVR and G2: non-SVR).

Plasma cytokine and chemokine levels [IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), RANTES, monokine induced by IFN gamma (MIG), and IFN gamma-induced protein 10 (IP-10)] were quantified at two time points of treatment. Comparative analyses were performed by evaluating the cytokine and chemokine levels between the two groups at the same time point (G1 × G2 – baseline; G1 × G2 – W12) and between the two treatment points within the same group (G1 – baseline × W12; G2 - baseline × W12).

Statistical tests were performed using SAS software version 4.0 for Windows. $P<0.05$ was considered statistically significant. Fisher's exact test was used for association analyses. The Kruskal-Wallis nonparametric test was used to compare the variables when the data did not satisfy normality assumptions.

For sample collection, venous blood was placed in two tubes containing ethylenediamine tetraacetic acid (EDTA) (5 ml each). Samples were centrifuged for 10 min at 300 *g*, and aliquots of plasma were placed in Eppendorf® microtubes and subsequently frozen at –80 °C.

Cytokine and chemokine levels were analyzed by flow cytometry following the CBA method using the kits CBA Human Chemokine (MCP-1, RANTES, IL-8, MIG, and IP-10) and Human IL-6 Flex Set.

Data were collected after obtaining informed consent from the patients. Informed consent was additionally obtained from a patient's legally authorized representative when the patient could not consent. The procedures were conducted in accordance with the Helsinki Declaration of 1975, which was revised in 1983. The present study was approved by the Ethics Committee of the Botucatu Medical School (Approval number of the ethics committee: 773.353).

RESULTS

A total of 31 patients were screened, out of which four were excluded because of the following reasons: two deaths, one interrupted treatment, and one patient was assigned to the F2 stage based on the METAVIR score.

Of the 27 remaining patients, 78% (21) were cirrhotic. The majority of patients were male (67%). The average age of the patients was 55.11±10.03. SVR, F4, and F3 were observed in 63%, 57% (12/21), and 83% (5/6) of all patients.

Results from the analysis of cytokine and chemokine levels in the TT baseline period (G1 vs. G2) are presented in **Table 1**. RANTES (p=0.04) and IL-6 (p=0.02) levels were found to be associated with SVR, and downregulation of both parameters was observed in the SVR group.

TABLE 1: Comparison of cytokine and chemokine levels between SVR and non-SVR patients undergoing triple therapy at the baseline period.

Variable (n=27)	SVR (n=17)	Non-SVR (n=10)	P-value
IP10	723.36 (601.00 – 1240.25)	1048.80 (752.30 – 1502.66)	0.37
MCP-1	24.95 (19.18 – 51.72)	39.34 (18.54 – 51.20)	0.76
MIG	335.48 (200.21 – 574.72)	336.02 (167.17 – 564.08)	0.76
RANTES	2306.08 (1629.33 – 2524.76)	2777.48 (2340.67 – 3440.24)	0.04*
IL8	15.02 (5.97 – 21.61)	21.10(11.78 – 40.10)	0.12
IL6	0 (0 – 0)	0.29 (0 – 4.61)	0.02*

SVR: virological response. Interleukin **(IL)-6, IL-8**, regulated on activation normal T cell expressed and secreted **(RANTES)**, monocyte chemotactic protein-1 **(MCP-1)**, monokine induced by gamma IFN **(MIG)** and IFN gamma-induced protein 10 **(IP-10)**. Results are expressed as a median and interquartile range (25; 75).

Table 2 shows the comparison of cytokine and chemokine levels between the G1 and G2 groups at the W12 time point. RANTES (p=0.04) and IL-8 (p=0.01) levels were downregulated in the G1 group, and the observed differences were significantly associated with SVR. Expression levels of other cytokines and chemokines were not associated with SVR, although some parameters were higher in the non SVR group.

Table 3 and **Table 4** show the comparison between the baseline and W12 time points in the G1 and G2 groups, respectively.

Table 3 (G1) shows significant differences in the plasma levels of IL-6 (p=0.02) and MCP-1 (p=0.001) between the two different time points. Both treatment groups showed the upregulation of IL-6 and MCP-1 levels at W12. Although other parameters showed similar patterns, the observed differences did not reach statistical significance. RANTES was the only parameter that was downregulated at W12 relative to W0; however, the observed difference was not statistically significant.

As shown in **Table 4,** the RANTES (p=0.05) and MCP-1 (p=0.01) levels were significantly different between the baseline and W12 time points in the G2 group. Although the other cytokines and chemokines showed no significant differences between the two time points, an increase in W12 levels occurs

DISCUSSION

Elucidating the mechanisms underlying IR during the different treatment regimens for HCV is of great importance because the SVR is influenced by the host IR, as previously established by studies on patients undergoing dual therapy[14,17,21,26]. In particular, previous findings indicated that the production of cytokines and chemokines plays an important role in the IR during HCV infection. However, there is limited knowledge on the IR during TT. Thus, there is a need to investigate whether the addition of PIs to PegIFN/RBV treatment alters cytokine and chemokine profiles and whether the induced changes enhance the host's response to the treatment.

Our results showed that circulating levels of RANTES and IL-6 at the baseline period were associated with SVR (**Table 1**). Patients who did not achieve SVR presented higher

TABLE 2: Analysis of cytokine and chemokine levels between SVR and non-SVR patients undergoing triple therapy at W12.

Variable (n=27)	SVR (n=17)	Non-SVR (n=10)	P value
IP10	764.25 (557.09 – 980.73)	1029.90(610.24 – 1476.23)	0.34
MCP-1	63.94 (53.29 – 121-87)	78.12 (50.69 – 119.01)	0.96
MIG	421.51 (266.19 – 872.92)	410.83 (225.43 – 499.41)	0.58
RANTES	2239.00 (1629.33 – 3756.25)	4003.89 (3381.49 – 4698-48)	0.04*
IL8	19.17 (11.58 – 30.41)	37.20 (23.04 – 56.83)	0.01*
IL6	0 (0 – 3.46)	2.19 (0 – 8.78)	0.59

SVR: virological response. Interleukin **(IL)-6, IL-8**, regulated on activation normal T cell expressed and secreted **(RANTES)**, monocyte chemotactic protein-1 **(MCP-1)**, monokine induced by gamma IFN **(MIG)** and IFN gamma-induced protein 10 **(IP-10)**. Results are expressed as a median and interquartile range (25; 75).

TABLE 3: Analysis of cytokine and chemokine levels in SVR patients at baseline and W12.

Variable (n=27)	Week 0	Week 12	P value
IP10	723.36 (601.00 – 1240.25)	764.25 (557.09 – 980.73)	0.89
MCP-1	24.95 (19.18 – 51.72)	63.94 (53.29 – 121-87)	0.001*
MIG	335.48 (200.21 – 574.72)	421.51 (266.19 – 872.92)	0.36
RANTES	2306.08 (1629.33 – 2524.76)	2239.00 (1629.33 – 3756.25)	0.38
IL8	15.02 (5.97 – 21.61)	19.17 (11.58 – 30.41)	0.26
IL6	0 (0 – 0)	0 (0 – 3.46)	0.02*

SVR: virological response. Interleukin **(IL)-6, IL-8**, regulated on activation normal T cell expressed and secreted **(RANTES)**, monocyte chemotactic protein-1 **(MCP-1)**, monokine induced by gamma IFN **(MIG)** and IFN gamma-induced protein 10 **(IP-10)**. Results are expressed as a median and interquartile range (25; 75).

TABLE 4: Analysis of cytokine and chemokine levels in non-SVR patients at baseline and W12.

Variable (n=27)	Week 0	Week 12	P value
IP10	1048.80 (752.30 – 1502.66)	1029.90(610.24 – 1476.23)	0.91
MCP-1	39.34 (18.54 – 51.20)	78.12 (50.69 – 119.01)	0.01*
MIG	336.02 (167.17 – 564.08)	410.83 (225.43 – 499.41)	0.62
RANTES	2777.48 (2340.67 – 3440.24)	4003.89 (3381.49 – 4698-48)	0.05*
IL8	21.10(11.78 – 40.10)	37.20 (23.04 – 56.83)	0.14
IL6	0.29 (0 – 4.61)	2.19 (0 – 8.78)	0.69

Interleukin **(IL)-6, IL-8**, regulated on activation normal T cell expressed and secreted **(RANTES)**, monocyte chemotactic protein-1 **(MCP-1)**, monokine induced by gamma IFN **(MIG)** and IFN gamma-induced protein 10 **(IP-10)**. Results are expressed as a median and interquartile range (25; 75).

IL-6 levels than those in the RVS group. Therefore, our findings were consistent with previous studies showing that the cytokine levels were associated with double therapy and that IFN influenced cytokine behavior[27,28]. Lower baseline RANTES levels were observed in the RVS group (**Table 1**), consistent with the findings reported in an independent study that investigated patients who received dual therapy[29]. However, another study[30] reported that high RANTES levels at baseline were associated with SVR in dual therapy in patients with low fibrosis (F≤2). On the other hand, our subjects included 78% cirrhotic patients and 22% advanced fibrosis patients.

Our current findings showed that patients with higher levels of IP-10 at baseline did not reach SVR and were not associated with SVR. Nevertheless, some studies indicated that low serum levels of IP-10 at baseline were associated with SVR during dual therapy[31-34].

Baseline IL-8 levels were higher in the non-SVR group compared to those in the SVR group, but the differences did not reach statistical significance. Studies have shown that increased serum IL-8 levels were associated with non-response to IFN-based treatment and disease progression[35,36].

Table 2 shows the analysis of IL-8 and RANTES levels with SVR at W12. The patient group with lower IL-8 levels reached SVR.

The chemokine IL-8 is an important member of the chemokine CXC family. The key function of IL-8 is to attract polymorphonuclear cells to sites of tissue injury and inflammation. IL-8 is synthesized by several cell types, including monocytes, macrophages, Kupffer cells, hepatocytes, and hepatic stellate cells[35-37]. IL-8 levels are upregulated in the peripheral blood and liver, thereby indicating that increased tissue infiltration and macrophage-induced hepatic activation are mediated by the interaction between IL-8 and CXCR-1 during hepatitis C infection[35].

RANTES levels were found to be associated with SVR in W12 (**Table 2**), similar to baseline levels (**Table 1**). These results were contradictory to those of a previous study on patients undergoing dual therapy,[29] in which RANTES levels were not associated with SVR, although RANTES levels were upregulated during treatment in patients who did not achieve SVR. Multiple studies reported different effects of IFN- α on RANTES expression in different cell types[38, 39].

More recently, two studies on patients subjected to TT evaluated the influence and behavior of IP-10 chemokines in SVR. One study evaluated 15 patients at different time points during the treatment period and did not identify differences in IP-10 chemokine levels between the SVR and non-SVR groups[40]. Another study evaluated 97 patients at baseline and showed that IP-10 levels were associated with SVR[41]. Our results showed no association between IP-10 and SVR in any of the evaluated time points. However, patients in the SVR group were observed to have lower IP-10 levels at both time points. Analysis of a larger sample size are likely to reproduce the above findings.

Median cytokine levels of patients in the SVR group were evaluated at baseline and W12 (**Table 3**). Notably, MCP-1 and IL-6 levels were significantly different between the two time points. A previous study assessed HIV co-infected patients and observed that high IL-6 levels were associated with therapeutic failure with IFN-α[42]. Another study showed that IL-6 levels were decreased during IFN-α treatment in patients who reached SVR. Similar changes were observed among non-SVR patients, although IL-6 levels were restored at the end of the dual treatment[43].

MCP-1 chemokines are known to play roles in the chemotactic recruitment of monocytes. Our current results showed that MCP-1 levels were upregulated with the independent response found in TT during treatment (**Table 3** and **Table 4**). A previous study reported similar findings during dual therapy treatment.[44] However, another study[45] indicated that the same pattern was observed only in SVR patients.

Table 4 compares the variables in the non-SVR group between the baseline and W12. Both MCP-1 and RANTES levels were significantly different between the two time points.

The chemokine RANTES was found to be significantly upregulated during treatment in the non-SVR group (**Table 4**) at the baseline and W12 time points. In accordance with a previous study, the observed increase in RANTES levels was associated with the presence of inflammation. The hepatitis C virus induces RANTES production by macrophages[46]. Evidence suggests that IFN type I inhibits the production of RANTES by T cells[47]. Therefore, consistent with the expected results, RANTES levels did not increase during TT treatment in the SVR group (**Table 3**).

A previous study that assessed cytokine and chemokine levels during dual therapy treatment showed that IL-8 levels were lower throughout the treatment and were associated with SVR[48]. In contrast to the abovementioned findings, our current results indicated that IL-8 levels were upregulated during the early periods of TT treatment in both patient groups (**Table 3** and **Table 4**).

In a previous study on patients undergoing dual therapy,[49] levels of the cytokine MIG were shown to be downregulated during SVR. By contrast, our results showed that the MIG levels were upregulated in both groups (Table 3 and 4); however, the SVR group presented higher MIG levels at W12, although the differences were also not significant (**Table 2**). Moura et al. (2011) investigated subjects undergoing dual therapy and showed that patients in the SVR group had higher MIG levels than those in the non-SVR group; however, the differences were not statistically significant.

CONCLUSION

Our findings showed that RANTES, IL-6, and IL-8 levels were associated with SVR during TT treatment. Our results were similar to those observed in patients with double therapy, thereby supporting the role of IFN in the host RI. Further studies are required to investigate the cytokine and chemokine profiles under treatment with currently available IFN-free drugs, which consequently induce different immunological and virologic mechanisms without the interference of IFN.

Conflict of interest

The authors declare that there is no conflict of interest.

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