Viral Hepatitis

Phenotypic features of innate and adaptive immunity in patients with chronic hepatitis C and end-stage renal disease

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Abstract

Background: The knowledge of the immunological profile of patients with chronic hepatitis C (CHC) and end-stage renal disease (ESRD) on haemodialysis (HD) is still limited. Aims: This study investigated the immune response profile in HCV patients with concomitant ESRD focusing on the influence of the renal disease on the phenotypic profile of peripheral blood lymphocytes. Methods: Immunophenotypic features of peripheral blood leucocytes were assessed by flow cytometry in two distinct groups: HCV patients with ESRD (CHC+ESRD, n = 16) and HCV patients with normal renal function (CHC, n = 20). Two control groups that were included were as follows: HCV negative blood donors (BD, n = 15) and HCV negative patients with ESRD (ESRD, n = 19). Results: Higher frequency of macrophage-like and pro-inflammatory monocytes along with enhanced frequency of CD3⁺ CD16⁺ CD56⁺ NK-cells, were the hallmark of CHC+ESRD patients. Lower frequency of B cells with significant decreased of B1 and CD23⁺ B-cells were associated with ESRD, regardless the HCV infection. Although higher rates of activated CD4⁺ and CD8⁺ T cells were observed in the CHC and CHC+ESRD groups, the chemokinesis of T-cell subsets, based on their chemokine receptor expression, was affected by ESRD. Conclusions: Chronic hepatitis C patients with ESRD on HD exhibit distinctive phenotypic profile of circulating leucocytes. It may be implicated in the natural history of HCV infection in this particular group of patients and warrants further investigation.

The immune system plays a critical role in the natural history of hepatitis C virus (HCV) infection. However, the knowledge of immunological aspects of the chronic hepatitis C (CHC), especially in patients with end-stage renal disease (ESRD), is still limited. The course of HCV infection, the most important cause of chronic liver disease in ESRD patients on regular haemodialysis (HD), has peculiar pathogenesis and natural history (1, 2). Most patients have normal ALT levels, lower rates of advanced fibrosis and cirrhosis, absence of correlation between ALT and histology, lower HCV-RNA levels and variable anti-HCV antibodies titers (1, 3).

There is a paucity of studies targeting the critical aspects of viral clearance and the immune status of patients with CHC and ESRD. As CHC has a wide range of clinical manifestations, from minimal hepatic lesions to cirrhosis, the factors influencing the nature and severity of disease progression need to be clarified. Distinct clinical and histological presentations of CHC have suggested an interaction of multiple viral and host factors and the combination of different immunologic parameters to explain the hepatic injury caused by HCV infection in ESRD patients. Hence, the better comprehension of the immune response patterns may help to explain the natural history of HCV infection and the diversity of clinical presentations of this particular group of patients (4, 5).

The immunophenotyping of peripheral blood has been employed in studies focusing on the pathogenesis of CHC. A correlation among some immunophenotypic profiles, liver inflammation, severity of liver fibrosis and therapeutic response have been suggested recently (6–8). Hence, we sought to investigate the profile of the immune response in CHC patients with ESRD on HD,
focusing on the major phenotypic features of peripheral blood lymphocytes.

Patients and methods

Study group

Seventy eligible patients from participant institutions were prospectively included. The groups and inclusion/exclusion criteria were established as follows: (a) CHC/ESRD patients ($n = 16$) on HD (CHC defined as above) (CHC+ESRD group); and (b) CHC patients ($n = 20$) treatment-naive, anti-HCV and HCV-RNA (AMPLICOR$^\text{®}$, Roche Molecular Systems, lower limit of detection of 50 IU/ml) positive, with normal renal function (CHC group). Control groups were as follows: (a) voluntary blood donors (BD group) [$n = 15, 8$ (56.3%) male patients, median age 31.4 years (range 19–50)]; and (b) ESRD patients on HD with anti-HCV negative (3rd generation ELISA), normal ALT, negative HCV-RNA-PCR (ESRD group) [$n = 19, 10$ (52.6%) male patients, median age 50.3 years (range 36–62)]; Table 1 shows the characteristics of the CHC and CHC+ESRD groups. Exclusion criteria were decompensated cirrhosis, HBV and/or HIV co-infection, concomitant autoimmune diseases, alcohol intake more than 20 g/day (female gender) and 40 g/day (male gender), and use of immunosuppressive drugs.

The protocol was approved by institutional and hospital Ethical Boards. All participants were required to give written informed consent.

Laboratory proceedings

Peripheral blood samples were collected from all participants and promptly analysed. EDTA-treated peripheral blood white cells were labelled with fluorochrome-conjugated specific monoclonal antibodies following immunofluorescence analysis by flow cytometry. Qualitative and quantitative tests (PCR, AMPLICOR$^\text{®}$, Roche Molecular Systems Inc., US kits in Hermes Pardini Laboratory, Belo Horizonte, Brazil) were used to confirm diagnosis of HCV infection and measure of HCV viral load. HCV genotype was performed by direct sequencing of a segment from the 5' UTR region. Histopathological analysis was based on the METAVIR score of grade of inflammation and stage of fibrosis (9).

Immunophenotyping of peripheral blood leucocytes

White blood cell phenotypes were analysed ‘ex-vivo’, following an immunofluorescence procedure recommended by Becton Dickinson$^\text{®}$ (Mountain View, CA, USA). Briefly, 100 µl aliquots of EDTA whole peripheral blood samples were mixed in 12 × 75 mm tubes with 5 µl of undiluted monoclonal antibodies (mAbs) specific for several cell-surface markers labeled with fluorochrome (fluorescein isothiocyanate, phycoerythrin and tricolour). The list of mAbs used in this study is provided in Table 2. The tubes were incubated in the dark for 30 min at room temperature. Following incubation, erythrocytes were lysed using 2 ml of fluorescence-activated cell sorter (FACS) Lysing Solution (Becton Dickinson Biosciences Pharmingen, San Diego, CA, USA). After incubation, the cells were washed twice with 2 ml of phosphate-buffered saline containing 0.01% sodium azide. Cell preparations were fixed in 200 µl of FACS fix solution (10 g/L paraformaldehyde, 1% sodium cacodylate, 6.65 g/L sodium chloride, 0.01% sodium azide). Cytofluorimetric data acquisition was performed with a Becton Dickinson FACScan instrument.

The immunophenotyping covered the following cellular populations and surface markers:

- Frequency of leucocyte subsets: Innate immunity: Monocytes - MØ (CD14, CD14, HLA-DR); Natural Killer – NK cells (CD3, CD16, CD56) and NKT lymphocytes (CD3, CD16, CD56); - Adaptive immunity: T-cell subsets (CD4, CD8) and B cells (CD19, CD5);
- Markers of cell activation: - Activated T cells (HLA-DR) and B cells (HLA-DR, CD23);

| Table 1. Demographic and laboratory characteristics of patients with chronic hepatitis C |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Variable | CHC group* | CHC+ESRD group† |
| n (%) | n (%) | Statistics |
| Male sex | 11 (55%) | 10 (62.5%) | $P > 0.05$ |
| Age (years) | 52.9 (33–65) | 41.4 (24–63) | $P > 0.05$ |
| ALT (IU/L) | 76.0 (17–132) | 30.5 (17–77) | $P = 0.002$ |
| Reference: male <45/ female <35 | | |
| HCV-RNA (x 10^3 IU/ml) | 461.7 (49,388.0) | 182.5 (23,049.0) | $P = 0.011$ |
| Genotype‡ | | |
| 1 | 13 (76.0) | 6 (70.4) | $P > 0.05$ |
| 2 | 1 (5.9) | 2 (11.1) |
| 3 | 3 (17.6) | 2 (18.5) |
| Grade of inflammation§ | | |
| 0 | 0 | 1 (8.3) |
| 1 | 12 (60.0) | 8 (66.7) |
| 2 | 6 (30.0) | 2 (16.7) |
| 3 | 2 (10.0) | 1 (8.3) |
| Fibrosis stage§ | | |
| 0 | 3 (15.0) | 3 (25.0) |
| 1 | 5 (25.0) | 5 (41.6) |
| 2 | 3 (15.0) | 2 (16.7) |
| 3 | 5 (25.0) | 0 |
| 4 | 4 (20.0) | 2 (16.7) |

*Chronic hepatitis C patients.
†Chronic hepatitis C and end-stage renal disease patients.
‡CHC – n = 17 and CHC+ESRD – n = 10.
§METAVIR score.
ALT, alanine aminotransferase.
Table 2. Monoclonal antibodies (mAbs) used for immunophenotypic analyses

<table>
<thead>
<tr>
<th>mAbs</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>FITC*</td>
<td>UCHT1</td>
<td>T cells</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>FITC*, PE†</td>
<td>RPA-T4.53.5</td>
<td>T-cell subset</td>
</tr>
<tr>
<td>Anti-CD5</td>
<td>FITC†</td>
<td>L17F12</td>
<td>B1-cells</td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>FITC*, TC†</td>
<td>RPA-T8, 3B5</td>
<td>T-cell subset</td>
</tr>
<tr>
<td>Anti-CD14</td>
<td>TC†</td>
<td>TUk4</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Anti-CD16</td>
<td>TC†</td>
<td>3G8</td>
<td>NK-cells, Monocytes</td>
</tr>
<tr>
<td>Anti-CD19</td>
<td>FITC*, PE†</td>
<td>4G7, SJ25-C1</td>
<td>B cells</td>
</tr>
<tr>
<td>Anti-CD23</td>
<td>PE*</td>
<td>M-L233</td>
<td>Activated B-cells</td>
</tr>
<tr>
<td>Anti-CD32</td>
<td>FITC*</td>
<td>FL8.26</td>
<td>B cells</td>
</tr>
<tr>
<td>Anti-CD56</td>
<td>PE*</td>
<td>B159</td>
<td>NK-Cells</td>
</tr>
<tr>
<td>Anti-HLA-DR</td>
<td>PE†</td>
<td>L17F12</td>
<td>T cells</td>
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<tr>
<td>Anti-CXCR3</td>
<td>FITC§</td>
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<td>Anti-CR3</td>
<td>FITC§</td>
<td>61828.111</td>
<td>T-cell subsets</td>
</tr>
</tbody>
</table>

*BD Pharmingen.  
†Caltag.  
§Serotec.  
€R&D Systems.  
FITC, fluorescein isothiocyanate; PE, phycoerythrin; TC, tricolour.

- Markers of immune response regulation:  
  - Expression of IgG receptor by B cells (CD32-FcγRII);  
  - Expression of chemokine receptors by T-cell subsets (CXCR4, CXCR3, CCR3).

Statistical analysis

The CELLQuestTM software provided by the manufacturer was used for data acquisition and analysis, through different phenotyping strategies based on proposals from the literature (10–12). Considering the nonparametric nature of all data sets, statistical analyses were performed by Kruskal–Wallis test, followed by the Dunn’s post-test. Significant differences were considered at P < 0.05 and highlighted in the figures.

Results

Analysis of Leucocyte subsets

Innate immunity

The CHC+ESRD group had the highest frequency of CD14+ MØ as compared to BD, CHC and ESRD groups. Higher rates of CD14+CD16+ macrophage-like and CD14+CD16+DR++ pro-inflammatory MØ were found in the ESRD and CHC+ESRD patients as compared to BD and CHC groups (Fig. 1A).

Despite no difference in the percentage of CD3–CD16++CD56++ NKT-cells among groups, higher frequency of CD3–CD16–CD56− activated NKT-cells was observed in the CHC+ESRD as compared to BD and ESRD groups (Fig. 1B). The analysis of the CD56 expression demonstrated that CHC+ESRD group has a higher frequency of CD3–CD16–CD56++ NKT-cells as compared to the other groups. Lower frequency of CD3–CD16–CD56− NKT-cells was observed in the CHC and CHC+ESRD patients as compared to BD and ESRD respectively (Fig. 1B). Although the frequency of total CD3+CD16−/CD56− NKT-cells was similar among the groups (P = 0.757), higher percentage of CD3+CD56+CD16− NKT-cell subsets was observed in the CHC and CHC+ESRD groups as compared to ESRD (Fig. 1B).

Adaptive immunity

The frequency of CD3+ T cells (P = 0.942), as well as the percentages of CD4+ (P = 0.496) and CD8+ T cells (P = 0.183) was similar among groups (Fig. 2A). However, the ESRD control group and CHC+ESRD patients presented significant lower frequency of CD19+ B cells as compared to BD and CHC (Fig. 2A). Likewise, lower rate of CD19−CD5+ B1-lymphocytes and complementary higher frequency of conventional B cells were observed in the ESRD control group and in CHC+ESRD patients as compared to BD and CHC (Fig. 2A).

Expression of cellular activation markers

T cells

The analysis of HLA-DR activation markers by CD4+ and CD8+ T cells showed higher frequency of CD4+HLA-DR++ (P < 0.01) and CD8+HLA-DR++ (P < 0.05) activated T cells in the CHC and CHC+ESRD patients as compared to the BD group (Fig. 2B).

B cells

The expression of the HLA-DR molecule by CD19+ B cells was similar among groups (P = 0.109). However, the expression of CD23 activation marker on B cells was significantly lower in the ESRD and CHC+ESRD groups as compared to BD (Fig. 2B).

Assessment of the immune response regulation

Expression of IgG receptor by B cells

The expression of CD32 (FcyRII) expression by CD19+ B cells was similar among the clinical groups (P = 0.754) (Fig. 2B).

Expression of chemokine receptors by T cells

The expression of CXCR4 by CD4+ T cells was lower in the CHC+ESRD group as compared to BD (Fig. 3A). Similarly, the expression of CXCR4 by CD8+ T cells was lower in the CHC+ESRD group as compared to BD (Fig. 3B).
lower in the CHC + ESRD group as compared to BD and ESRD groups (Fig. 3B).

Higher expression of CXCR3 by CD4+ T cells was observed in the ESRD control group as compared to CHC patients. On the other hand, lower CXCR3 expression by CD8+ T cells, was observed in the CHC + ESRD as compared to BD and ESRD control groups (Fig. 3B).

The expression of CCR3+ expression was reduced in CD4+ (Fig. 3A) and CD8+ T cells (Fig. 3B) in CHC patients as compared to BD.

**Discussion**

The major objective of this investigation was to demonstrate that CHC and CHC + ESRD patients present a distinct pattern of immune response as a result of complex mechanisms involving innate and adaptive immunity. The importance of this lies in the fact that this peculiar immune pattern may be associated with the induction and/or regulation of the pathogenesis HCV infection in ESRD patients (13, 14).
Fig. 2. Phenotypic profile of adaptive immunity cells in the peripheral blood. (A) lymphocyte subsets; (B) activation status of T and B cells. BD, blood donors; ESRD, end-stage renal disease; CHC, chronic hepatitis C; CHC+ESRD, chronic hepatitis C and end-stage renal disease.
The analysis of immunological features demonstrated a relevant change in the innate immunity compartment in CHC+ESRD patients. Macrophage-like and pro-inflammatory monocytes (15) were higher in the ESRD and CHC+ESRD patients, being the higher frequency of pro-inflammatory MØ observed in CHC+ESRD group. Renal failure is believed to impact on the number and activation of phagocytic cells. Studies of the immune response and cytokine production in haemodialysis patients have shown that the blood/membrane interaction may activate mononuclear cells, inducing cytokine production and generating a chronic inflammatory state in the bloodstream (16–18), which could be even higher in CHC+ESRD patients. In addition, the CHC+ESRD group showed higher levels of CD3−CD16−CD56+ NK-cells, a phenotype of putative activated cells with higher lytic activity (13). NK-cells are the predominant population of the innate immunity in the healthy liver (14, 19). Intra-hepatic lymphocytes and NK-cells do not undergo local clonal expansion, but migrate from peripheral extra-hepatic sites to the chronically infected organ. (20). NK-cells are essential effectors of the immune response against viral agents and are responsible for the antibody-dependent cytotoxic activity as well as interaction with dendritic and T cells, being an important link between innate and adaptive immunity (21, 22). HCV is known to evade the immune response mediated by NK-cells (23). Approximately 90% of NK-cells are CD56dim and express high levels of FcγRIII (CD16), while around 10% are CD56bright. CD56dim NK-cells have higher intrinsic cytotoxic activity and lower IFN-γ production, whereas CD56bright NK-cells have exactly the opposite characteristics (24). Our results showed an increase in the CD56dim NK-cell population in CHC+ESRD patients. The innate immunity profile of the CHC+ESRD group, with higher rates of activated NK-cells, and of NK-cells, might contribute to a particular pattern of immune response.

The CHC and CHC+ESRD groups had higher rates of CD3+CD16−CD56+ NKT-cells, which account for one-third of the hepatic CD3+ T cells and, functionally, seem to have a role in tumoural surveillance and autoimmunity phenomena (14, 25). Knowledge about the role of innate immunity in the progression of HCV infection is limited to only recent researches (13, 26, 27). Hence, we highlight the importance of this study as a comprehensive analysis of the respective components.

The analysis of adaptive immunity demonstrated that CHC and CHC+ESRD groups presented higher levels of activated CD4+HLA-DR+ and CD8+HLA-DR+ T cells, reflecting the recognized cellular activation in the context of CHC, regardless of the presence of ESRD (28).
The influence of the humoral immune response on the natural history of HCV infection has not been clearly defined (4). The presence of antibody does not seem to correlate with protection (29). Conversely, recent research with hepatic tissue cultures has identified antibodies that block the entry of HCV into hepatocytes, through an in vitro neutralization of viral particles (30). In this study, there was a clear reduction in the frequency of B cells in ESRD and CHC+ESRD patients, mainly of CD19+CD5+ B1-lymphocytes, which represent a B-cell subset predominantly involved with autoimmunity phenomena and IgM production (31). Despite the expression of CD32 (FcγRII) (32) and HLA-DR by B cells being similar among groups, the expression of the CD23 activation marker, which consists of an IgE receptor in CD19+ B-cells, was lower in the ESRD and CHC+ESRD groups. Some studies have shown that the cellular immune response is relatively preserved in ESRD, with concomitant deficiency of the humoral immune response (1, 33). Recent study concluded that the viral persistence in dialysis patients is due to a failure of the adaptive immune system, as shown by the absence of significant T-cell and antibody responses, as well as viral variability (34).

The chemokines (35–37) are important in CHC, as the intra-hepatic lymphocyte pool results from continuous migration (21). Our data demonstrated that CD4+ and CD8+ T-cell subsets from CHC+ESRD displayed decreased expression of CXCR4 molecule, responsible for the chemotaxis of naive T-lymphocytes (38). Moreover, higher rate of CD4+CXCR3+ T cells occurred in the ESRD control group. We also observed decreased number of peripheral CD8+ T cells expressing CXCR3+ (responsible for the chemotaxis of activated T-lymphocytes) in ESRD, a finding that underscores the importance of intra-hepatic recruitment of CD8+HLA-DR+ for the control of HCV chronic infection. The CD8+ CXCR3 lymphocytes have been associated with the control of HCV viremia, influencing the recruitment of CD8+ T cells, and the hepatic inflammation (8).

The analysis of the expression of CCR3+ in CD4+ and CD8+ T cells showed decreased levels in the CHC group. In a global analysis, we found out that the CHC group is characterized by a predominantly cellular immune response, particularly characterized by a decreased expression of CCR3 in T cells. Recent evidence points to the close relation between the expression of this chemokine receptor and the severity of the hepatic necroinflammatory activity (6, 8). The chemokine receptor expression pattern may be used as an indicator of distinct patterns of immune response (37). Accordingly, effector T cells express different chemokine receptors, which determine the type of the immune response, influencing the outcome of HCV infection, that is virus clearance or persistence, as well as the severity of liver tissue inflammation (39, 40).

In summary, we have demonstrated here that CHC+ESRD patients present a global change in their innate immunity compartment, predominantly in MØ activation markers and NK-cell subsets. Hence, we suggest that monocyte activation seems to be related to ESRD, which is in agreement with previous reports (17, 41). CHC+ESRD group had higher rates of activated NK-cells and NK-cells with higher cytolytic potential (CD56bright CK) and lower CD56dim NK-cells. In chronic HCV infection, cytolytic NK-cells have a protective role, while high levels of IFN-γ (produced by CD56bright NK-cells) are responsible for the hepatic injury, offsetting the control of HCV infection (42). In addition, it was noteworthy the decreased levels of circulating B-cells, specially the B1-cell subset, in ESRD and CHC+ESRD patients. These findings, along with the decreased levels of CD23+ activated B-cells are consistent with the impaired functional activities of the humoral immune response observed in patients with end-stage renal disease. A reduction in chemokine receptor expression in CD4+ and CD8+ T cells was noted in the CHC+ESRD group, chiefly CXCR4 and CXCR3 by CD8+ T cells, suggesting that the cell chemotaxis is affected in ESRD and CHC+ESRD, which can be directly related to the cell migration and recruitment to the liver. These results strongly suggest that CHC patients with ESRD patients have a robust innate immunity activation and deficient adaptive immune response, which may impact on the degree of inflammatory hepatic injury. The analysis of immunological parameters put forward new perspectives in the evaluation of CHC in patients with concomitant ESRD. Further research into the profile of plasma soluble cytokines and comparative analysis of the phenotypic parameters of the hepatic compartment is warranted.

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