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ORIGINAL INVESTIGATION

Influence of Hepatitis C virus coinfection on immune reconstitution in HIV subjects

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Abstract
Despite successful HIV suppression by antiretroviral treatment (ART), immune activation may persist in HIV patients, contributing to an impaired immunological reconstitution and disease progression. Information regarding Hepatitis C virus (HCV) coinfection as a factor that accounts for immune activation in HIV subjects remains unclear. Furthermore, most studies have been carried out considering HIV/HCV patients as a whole, without taking into account the presence or absence of liver damage. Therefore, it is unknown if HCV and/or its liver-related disease could act as two independent factors contributing to the immune activation. In this study, we investigated the presence of immune activation in a cohort of 50 HIV/HCV patients by measuring cytokine levels, $CD4^+$ T-cell counts and CD4/CD8 ratios. Six patient groups were defined according to HIV viral load, HCV status, and liver disease to assess the impact of each of these factors on immune activation and reconstitution in HIV/HCV patients. Only subjects with controlled HIV infection and cleared HCV displayed immunological parameters within normal ranges. The mere presence of HCV contributes to immune activation leading to an inappropriate immunological reconstitution. This state exacerbates in the presence of HCV-associated liver disease. Our results suggest that ART is not enough to suppress immune activation in the context of HIV/HCV coinfection, since both HCV and its liver-related disease would contribute to the immune activation. Given that immune activation worsens immunological reconstitution and clinical status, these results support the priority of HCV treatment in HIV/HCV patients and suggest the monitoring of their liver status.

Keywords HIV/HCV coinfection · Antiretroviral treatment · Immune activation · Liver disease · Immunological reconstitution

Introduction

Generalized immune activation is one of the hallmarks of HIV infection. Basically, every component of the immune system is in a hyperactive state during chronic untreated HIV infection. Non-specific T-cell activation and proliferation, increased T-cell turnover, polyclonal B-cell activation, and elevated plasma levels of proinflammatory cytokines are characteristics of this phase. Most important, T-cell activation is associated with $CD4^+$ T-cell depletion, sustained viral replication, and HIV disease progression. Different mechanisms are thought to contribute to the generalized immune system activation including persistent HIV replication, bacterial translocation associated with the loss of gut barrier integrity, and the presence of other coinfections [1–4]. Antiretroviral treatment (ART) hits directly on HIV replication, thus removing one of the main sources of stimuli for immune activation. In general, suppression of plasma HIV viral load by ART is followed by immunological reconstitution usually noted as an increase in the circulating $CD4^+$ T-cell counts [5]. However, even after several years of sustained plasma HIV viral load suppression, some patients show an insufficient $CD4^+$ T-cell count recovery, reaching

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sometimes a plateau. The persistence of immune activation despite suppressive ART would seem to be involved in the failure of immune reconstitution [3]. The CD4/CD8 ratio is a well-established predictor of progression to AIDS in untreated HIV infection [6]. Besides, in treated HIV patients, the CD4/CD8 ratio often fails to normalize and it has also been linked to immune activation [7].

Given shared modes of transmission, coinfection with Hepatitis C virus (HCV) and HIV is relatively common [8]. As HIV disease, HCV infection generally courses as asymptomatic disease for several years, but also could lead to the establishment of chronic liver disease which can range from mild to severe, including cirrhosis and liver cancer [9]. HIV/HCV-coinfected individuals have increased morbidity and mortality, even in the ART era [10]. Many studies suggest that HIV infection accelerates HCV-mediated liver pathology; however, it is less clear the information about the impact of HCV infection on HIV disease progression [8, 10–13]. There are also conflicting data regarding the role of HCV coinfection as a pathogenic factor related to the inappropriate immune reconstitution in HIV viral suppressed subjects [14–18]. Moreover, most of these studies have been carried out considering HIV/HCV coinfected subjects as a whole, without taking into account the presence or not of liver-related disease. Therefore, the information about the presence of HCV and its associated liver disease as two independent factors that may contribute to immune activation in HIV subjects is limited.

In this study, we evaluated the presence of immune activation in a cohort of patients with hemophilia and HIV/HCV coinfection, who have acquired both infections more than 20 years ago and developed different disease outcomes. For this purpose, we analyzed cytokine plasma levels, $CD4^+$ T-cell counts, and CD4/CD8 ratios in six different defined patient groups, to investigate the individual contribution of HIV viral load, HCV status, and liver disease on immune activation and reconstitution in HIV/HCV patients.

Materials and methods

Patients and study design

Fifty subjects from a cohort of patients with hemophilia who have acquired HCV and HIV viruses through contaminated clotting-factor concentrates and who were followed longitudinally for many years were included in the present study. All patients were males with inherited hemophilia and received medical care at the Fundación de la Hemofilia. To investigate the levels of immune activation and reconstitution in the study cohort, a cross-sectional analysis was conducted by selecting from each patient plasma samples with retrospective clinical data available. In addition, a second plasma sample belonging to stages before ART and with available retrospective clinical data was chosen from each patient to compare the state of immune activation before (pre) and after (post) the implementation of ART. According to HIV viral load, HCV status, and liver damage, patients were grouped as G1: virologically suppressed HIV subjects with cleared HCV ($n=7$), G2: virologically suppressed HIV subjects with chronic HCV infection without hepatic damage ($n=11$), G3: virologically suppressed HIV subjects with chronic HCV infection and hepatic damage ($n=10$), G4: subjects with detectable HIV viral load and cleared HCV ($n=4$), G5: subjects with detectable HIV viral load and chronic HCV infection without hepatic damage ($n=11$), and G6: subjects with detectable HIV viral load and chronic HCV infection with hepatic damage ($n=7$). A control group comprised by healthy blood donors and non-infected patients with hemophilia, all males and with age comparable to infected patients was included (C, $n=20$). Written informed consent was obtained from each participant. The study was approved by the local Ethics Committee of the Academia Nacional de Medicina and was conducted in accordance with the ethical principles of the 1975 Declaration of Helsinki.

Definitions and considerations

Virologically suppressed HIV subjects were defined as those on stable ART for at least 1 year, reaching and maintaining undetectable HIV viral load (< 50 copies/ml). Individuals with detectable HIV viral load included those untreated and those who showed poor adherence to ART, interrupting it. Chronic HCV infection was defined by the presence of antibodies against HCV and persistent HCV viremia (more than 6 months of detectable HCV-RNA). HIV/HCV seropositive individuals with cleared HCV included spontaneous resolvers and those who achieved a sustained virologic response after interferon-based treatment, as indicated by HCV antibody positivity and RNA negativity. Time points studied were > 12 months after spontaneous HCV clearance or the end of antiviral therapy.

The current recommendations propose to combine two non-invasive methods (e.g., transient elastography plus a biochemical index) to assess liver disease progression with the aim of increase diagnostic efficacy and avoid liver biopsy in as many cases as possible. However, in resource-limited countries (such as Argentina), the World Health Organization (WHO), the European (EASL), and the American (AASLD and AAEEH) guidelines recommend to evaluate the degree of liver fibrosis through the use of APRI (AST-to-Platelet Ratio Index) or FIB-4 indexes [19].

Therefore, as patients with hemophilia have not undergone liver biopsy routinely in Argentina and no transient elastography data were available by the study date, we

used the biochemical indexes APRI, FORNS, and FIB-4 to assess liver disease progression. We also considered the presence of clinical signs/symptoms characteristic of cirrhosis (hepatomegaly, splenomegaly, hepatosplenomegaly, esophageal varices, hepatorenal syndrome, liver failure, and portal hypertension) along with an AST/ALT ratio ≥ 1, which has been reported to have a good predictive value for advanced fibrosis or cirrhosis [20]. APRI, FORNS, and FIB-4 scores were calculated according to published formulas and used with the cut-offs recommended in bibliography (APRI < 0.5: absence of fibrosis, > 1.5: significant fibrosis, > 2: cirrhosis; FORNS < 4.2: absence of fibrosis, > 6.9: significant fibrosis; FIB-4 < 1.45: exclude severe fibrosis, > 3.25: confirm significant fibrosis) [21–23]. Patients showing two or more liver fibrosis indexes with values above the higher cut-off levels or 1 altered fibrosis index together with clinical signs of cirrhosis for at least 1 year of evolution were considered to present liver damage. Likewise, individuals with normal fibrosis indexes who maintained fibrosis indexes values below the higher cut-off levels since the acquisition of both infections were considered not to present hepatic damage.

Seroconversion dates for HCV and HIV infections are not exactly known, but are estimated to have occurred between 1975 and 1985 as commercial factor concentrates were not accessible in Argentina until 1975 and heat-inactivated concentrates were not available until late 1985. As it is presumed that HCV infection in individuals with hemophilia was acquired with the first clotting-factor exposure [24], we considered that patients born before 1975 became HCV infected in 1975. For those born after 1975, we estimated that HCV seroconversion took place within the first year of life. Dates of HIV seroconversion were based on previously published studies establishing 1982 as median year of seroconversion for hemophilia A and 1983 for hemophilia B [25].

Laboratory data

Data regarding the hepatic enzymes [serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (γ-GT)], cholesterol, platelet counts, type and severity of hemophilia, and death cause were obtained from medical records. HCV genotype, HIV and HCV viral loads, and serological data were performed and/or also collected from medical files. Determination of $CD4^+$ and $CD8^+$ T-cell counts (cells/mm^3) was performed by flow cytometry using the CellQuest software (BD Immunocytometry Systems, San Diego, CA, USA). A CD4/CD8 ratio ≥ 1.0 was considered normal. We recorded those laboratory measurements whose dates were closest to those of the plasma samples tested for immune activation.

Cytokine assays

Plasma isolated from EDTA anticoagulated blood was stored in aliquots at − 80 °C until used. Concentrations of IL-10, TGF-β, IFN-γ, and IL-2 were measured by ELISA using commercially kits according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Each plate included a standard curve of the corresponding recombinant human cytokine. Samples were assayed in duplicate and the results were expressed in pg/ml. Detection limits of the kits were 2 pg/ml for IL-10, 62 pg/ml for TGF-β and 4 pg/ml for IL-2 and IFN-γ. Values below the detection limit were recorded as the detection limit of the respective cytokine kit.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test were performed to assess differences among groups. Unpaired *t* test or Mann–Whitney test was used to compare immunological variables between two groups or between the pre- and post-ART stages. Qualitative variables were compared by the Chi-square test or Fisher's exact test. In all cases, a value of $p < 0.05$ was considered indicative of a significant difference. Data were analyzed using the GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

Results

Characteristics of HIV/HCV–coinfected patients

Six patient groups defined by HIV viral load, HCV status, and liver disease were studied with the aim to evaluate the impact of each of these factors on immune activation and reconstitution in HIV/HCV subjects. The clinical features of the studied groups are shown in Table 1.

Cytokine plasma levels

We analyzed the levels of the immunoregulatory cytokines IL-10 and TGF-β, and the levels of the proinflammatory cytokines IFN-γ and IL-2 in all groups. Immunoregulatory cytokine levels were similar among G1, G2, and C, but were significantly higher within G3–G6 (Fig. 1a, b). No significant differences were observed in the proinflammatory cytokine levels between G1 and C. An increase in the proinflammatory cytokine levels was seen within G2, but it was not statistically significant by ANOVA analysis. However, when these values were compared by a *t* test, significant differences in the proinflammatory cytokine levels were found between C and G2 (IL-2: $p = 0.01$; IFN-γ: $p = 0.02$) or between G1 and G2 (IL-2: $p = 0.05$;

Table 1 General characteristics of the HIV/HCV-coinfected patients

Variable	G1 ($n=7$)	G2 ($n=11$)	G3 ($n=10$)	G4 ($n=4$)	G5 ($n=11$)	G6 ($n=7$)	p
General characteristics							
Age (years)	37 (26–52)	35 (22–57)	41 (18–50)	26 (24–34)	27 (21–66)	46 (37–52)	0.1
Hemophilia A Se/Mo/Mi (n)	2/2/2	5/2/1	3/5/1	2/1/0	3/3/2	2/3/1	0.94
Hemophilia B Se/Mo/Mi (n)	0/1/0	2/1/0	1/0/0	0/1/0	0/1/2	0/0/1	
HIV-related characteristics							
HIV viral load (log copies/ml)	< 1.69 (< 1.69)	< 1.69 (< 1.69)	< 1.69 (< 1.69)	5.4 (4.8–5.9)	4.2 (3.1–5.2)	4.7 (2.5–6.2)	< 0.0001
Duration of viral suppression (years)	9 (2–15)	7 (1–15)	5 (1–14)	NA	NA	NA	0.50
Estimated duration of HIV (yrs)	27 (20–30)	23 (17–32)	22 (18–29)	20 (18–26)	22 (18–28)	21 (16–29)	0.21
HCV-related characteristics							
HCV viral load (log IU/ml)	< 1 (< 1)	5.9 (5.1–7.2)	5.7 (4.0–6.9)	< 1 (< 1)	5.9 (5.2–6.5)	5.7 (5.0–6.4)	< 0.0001
Estimated duration of HCV (years)	26 (20–33)	30 (22–39)	30 (18–36)	19 (18–22)	27 (20–32)	28 (23–36)	0.03
HCV genotype 1/others (n)	NA	9/2	6/3[a]	NA	6/5	6/1	0.41
ALT (IU/L)	34 (20–38)	49 (25–88)	88 (16–155)	38 (18–39)	41 (30–68)	51 (42–113)	0.0007
AST (IU/L)	26 (15–34)	35 (24–57)	111 (56–291)	37 (25–38)	34 (26–54)	102 (43–205)	< 0.0001
Liver status							
APRI score	0.3 (0.1–0.4)	0.4 (0.2–0.5)	2.5 (1.0–10.4)	0.4 (0.3–0.4)	0.5 (0.2–0.5)	1.5 (1.0–5.0)	0.0002
FORNS score	3.1 (1.7–4.6)	3.8 (0.2–5.3)	8.0 (5.0–10.4)	3.0 (2.2–3.4)	3.9 (2.4–5.8)	7.9 (7.5–8.7)	< 0.0001
FIB-4 score	0.5 (0.3–1.3)	0.9 (0.3–1.6)	4.5 (1.0–16.0)	0.6 (0.5–0.8)	0.7 (0.4–2.0)	4.4 (3.2–8.6)	< 0.0001
Time with scores altered (years)	NA	NA	6 (1–11)	NA	NA	5 (1–6)	NA
AST/ALT ratio	0.8 (0.5–1.0)	0.8 (0.6–1.3)	1.3 (0.7–5.1)	0.8 (0.6–2.0)	0.8 (0.5–1.2)	1.6 (1.0–2.0)	< 0.01
± Clinical signs of cirrhosis (n)	NA	NA	8/2	NA	NA	4/3	0.60

Values are expressed as median (range) unless otherwise noted. The p values from ANOVA, Chi-square, or Fisher's exact tests are indicated

G1: virologically suppressed HIV subjects with cleared HCV, G2: virologically suppressed HIV subjects with chronic HCV infection without liver damage, G3: virologically suppressed HIV subjects with chronic HCV infection and hepatic damage, G4: subjects with detectable HIV viral load and cleared HCV, G5: subjects with detectable HIV viral load and chronic HCV infection without hepatic damage, and G6: subjects with detectable HIV viral load and chronic HCV infection with liver damage

Se severe, *Mo* moderate, *Mi* mild, *NA* not applicable, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *APRI* AST-to-Platelet Ratio Index

[a]Genotype from one patient could not be obtained

IFN-γ: $p=0.008$), indicating that G2 displayed increased proinflammatory cytokine levels. Instead, no significant differences in the proinflammatory cytokines values were observed between C and G1 when they were compared by a t test (IL-2: $p=0.6$; IFN-γ: $p=0.7$), confirming that proinflammatory cytokine levels were similar between these groups. Otherwise, G3–G6 displayed significantly higher values of proinflammatory cytokines than C and G1 (Fig. 1c, d). These results indicated that only patients with controlled HIV infection and cleared HCV (G1) showed cytokine levels within the normal ranges. A switch towards a proinflammatory cytokine profile was preferentially seen in the presence of HCV (G2). However, liver pathology associated with HCV infection (G3) seems also to increase the amounts of immunoregulatory cytokines. On the other hand, subjects with uncontrolled HIV replication (G4–G6) displayed increased levels of both proinflammatory and regulatory cytokines.

$CD4^+$ T-cell counts and CD4/CD8 ratios

We next measured the levels of peripheral $CD4^+$ T cells among the groups. No significantly differences were observed in $CD4^+$ T-cell counts between C, G1, and G2. However, G3–G6 showed significantly decreased $CD4^+$ T-cell levels compared to C and G1. $CD4^+$ T-cell counts within G4–G6 were also significantly lower compared to G2 (Fig. 2a). CD4/CD8 ratios were comparable among G1 and C. G1 displayed significantly higher CD4/CD8 ratios than G2–G6. No significant differences were observed between G2 and G3, but a significantly higher CD4/CD8 ratio was observed within G2 as compared to G4–G6 (Fig. 2b). These data suggest that ART is necessary but not enough to increase $CD4^+$ T-cell counts to normal levels when HCV-associated liver disease is present (G3). Besides, the mere presence of HCV seems to be enough to contribute to immune activation, since patients on stable ART with

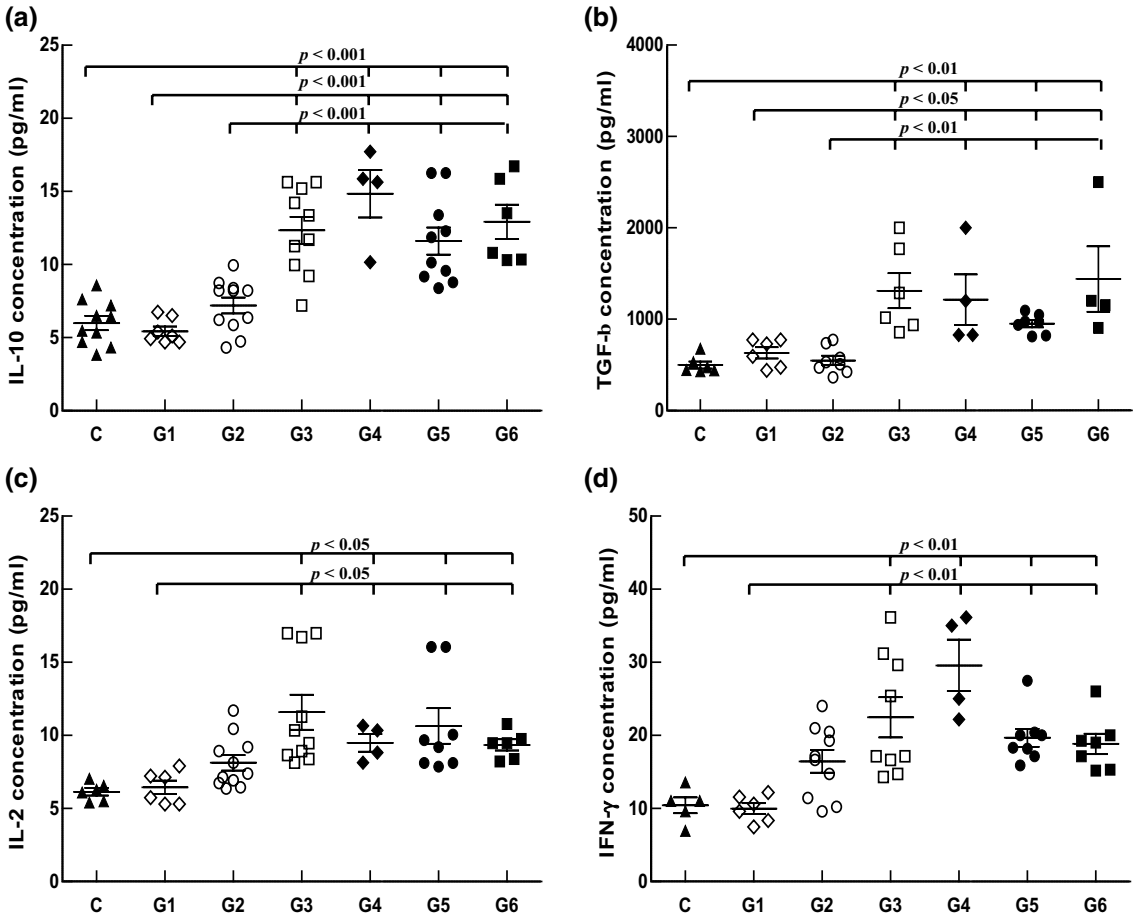


Fig. 1 Comparison of cytokine levels between the study groups. Plasma concentrations of the immunoregulatory cytokines IL-10 (**a**) and TGF-β (**b**) and the proinflammatory cytokines IL-2 (**c**) and IFN-γ (**d**) were detected by ELISA. *p* values were determined by one-way ANOVA followed by Tukey's multiple comparison test. Means ± SEM are indicated. C: control group, G1: HIV suppressed subjects with cleared HCV, G2: HIV suppressed subjects with chronic HCV infection, G3: HIV suppressed subjects with chronic HCV infection and hepatic damage, G4: HIV subjects with cleared HCV, G5: HIV subjects with chronic HCV infection, and G6: HIV subjects with chronic HCV infection and liver damage

chronic HCV infection without hepatic damage (G2) failed to normalize CD4/CD8 ratios. As expected, subjects with no control of HIV replication (G4–G6) showed the lowest $CD4^+$ T-cell counts and CD4/CD8 ratios, independently of the HCV presence or its liver-related disease (Fig. 2a, b).

Comparison between pre- and post-ART stages

To evaluate the influence of ART on immune activation and reconstitution, we compared the levels of cytokines, $CD4^+$ cells, $CD8^+$ cells, and CD4/CD8 ratios before and after the implementation of ART. At pre-ART stages, all groups displayed liver fibrosis indexes with values below the higher cut-off levels, suggesting that, at that time, no liver damage was present (data not shown). Before ART, all groups displayed high cytokine levels. After ART, only G1 significantly decreased the levels of IL-10, IL-2, TGF-β, and IFN-γ at levels similar to those of the control group. G2 also showed a reduction in IL-10 and TGF-β levels. Cytokine values in G3–G6 were basically unchanged between pre- and post-ART stages (Fig. 3a–d). Since G4–G6 included individuals untreated as well as those who stopped ART, these results depict the importance of ART to achieve normal cytokine levels. However, the fact that G2 (subjects on stable ART) failed to normalize IL-2 and IFN-γ levels suggests that continuous HCV presence could be affecting cytokine reversion to normal levels. This state would seem to be exacerbated when HCV-mediated liver pathology is present, as HIV/HCV subjects on stable ART with hepatic damage (G3) also displayed increased immunoregulatory cytokine levels. A significant increase in $CD4^+$ T-cell counts at post-ART stages was observed in G2 and G3 when they were compared to pre-ART stages (Fig. 3e). No significant changes were observed within G4–G6. Interestingly, G1 displayed normal $CD4^+$ T-cell counts before ART, which

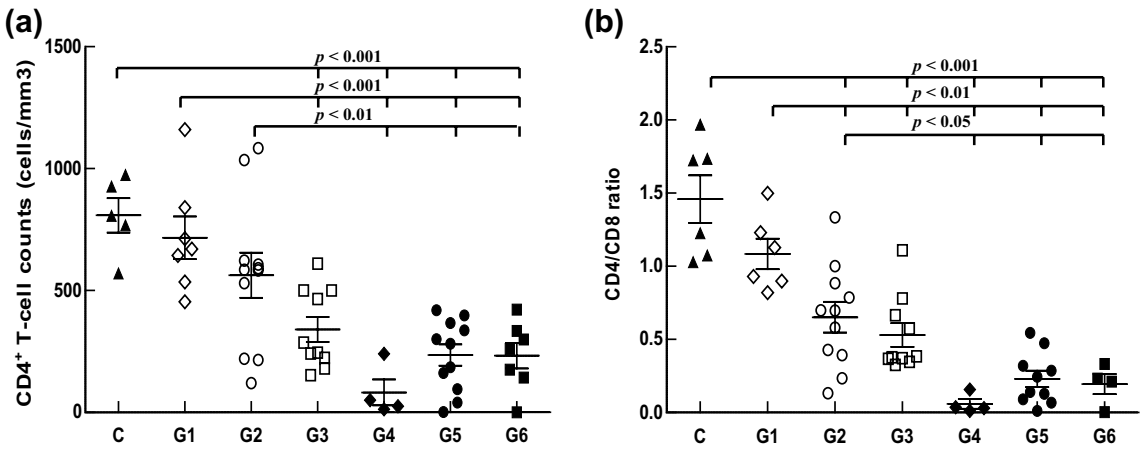


Fig. 2 CD4 + T-cell counts and CD4/CD8 ratios among the studied groups. CD4 + T-cell levels (**a**) and CD4/CD8 ratios (**b**) were determined by flow cytometry. *p* values were determined by one-way ANOVA followed by Tukey's multiple comparison test. Means ± SEM are indicated. C: Control group, G1: HIV suppressed subjects with cleared HCV, G2: HIV suppressed subjects with chronic HCV infection, G3: HIV suppressed subjects with chronic HCV infection and hepatic damage, G4: HIV subjects with cleared HCV, G5: HIV subjects with chronic HCV infection, and G6: HIV subjects with chronic HCV infection and liver damage

were maintained at post-ART stages (Fig. 3e). $CD8^+$ T-cell counts were high in all groups before ART. Only G1 significantly diminished $CD8^+$ cell levels at post-ART stages (Fig. 3f). Regarding CD4/CD8 ratios, a significant increase was observed in G1–G3 after the implementation of ART. However, a normal CD4/CD8 ratio was only reached by G1 (Fig. 3g). The gain in $CD4^+$ cell levels in G2 and G3 seems to be responsible for the increase in the CD4/CD8 ratios within these groups. Instead, the reduction of $CD8^+$ T-cell counts within G1 appears to be accountable for the normalization of CD4/CD8 ratios, which is consistent with the decrease of immune activation seen in this group. On the other hand, low CD4/CD8 ratios were maintained within G4–G6 (Fig. 3g), indicating that the immune activation present since early stages of HIV infection persisted among these groups.

Disease progression and clinical outcomes

Finally, we analyzed the clinical outcomes of the HIV/HCV coinfected patients by studying the percentages of death and its causes among each group. To date, all patients within G1 and G2 are alive. 60% of patients within G3 subsequently died, of them 50% was due to liver disease progression. Within G4, 75% of patients died, with AIDS-related complications being the cause. Of the 55% of patients within G5 who afterward died, 83% was due to AIDS-related complications. Finally, all patients belonging to G6 successively died. Of them, 43% of deaths were related to chronic liver disease and 29% to HIV/AIDS complications (Fig. 3h).

Discussion

Before ART, AIDS was the primary cause of death in HIV-infected patients. Nowadays, ART has been proven to successfully control HIV replication decreasing the incidence of AIDS and increasing the average life expectancy [26, 27]. However, in the ART era, life expectancy in HIV-infected patients is still lower than in uninfected persons, and mortality is related to a series of conditions generally associated with aging [26–31]. Ongoing immune activation and inflammation in treated HIV infection are proposed as the major driving forces of the accelerated immunological and systemic aging in HIV infection [31, 32]. Different conditions are thought to contribute to immune activation including increased microbial translocation due to impaired mucosal barrier integrity, the presence of coinfecting pathogens and HIV persistence in reservoirs [1–3, 31, 32].

In the current work, we evaluated the presence of immune activation in six different defined groups of HIV/HCV patients with more than 20 years of disease evolution, by analyzing cytokine levels, $CD4^+$ T-cell counts, and CD4/CD8 ratios. We observed that the group of HIV/HCV patients with controlled HIV infection and cleared HCV (G1) displayed normal cytokine levels along with the highest $CD4^+$ T-cell counts and CD4/CD8 ratios. Despite normal CD4 counts, a preferential proinflammatory cytokine profile together with an altered CD4/CD8 relation was seen in the group of HIV/HCV subjects on stable ART without liver disease (G2), while increased proinflammatory and immunoregulatory cytokine levels together with low $CD4^+$ T-cell counts and CD4/CD8 ratios were seen in the groups comprised by HIV/HCV subjects on stable ART with hepatic

damage (G3) and by HIV/HCV patients with uncontrolled HIV replication (G4–G6). These results indicate that only HIV subjects on stable ART and cleared HCV (G1) could achieve a proper immunological reconstitution. Instead, the presence of HCV by itself seems to be enough to contribute to immune activation, since HIV/HCV subjects on stable ART without liver disease (G2) failed to normalize proinflammatory cytokine levels and CD4/CD8 ratios. Furthermore, HCV-mediated liver pathology appears to exacerbate this state of immune activation by also increasing immunoregulatory cytokine levels and decreasing $CD4^+$ T-cell counts and CD4/CD8 ratios in HIV virologically suppressed and HCV-coinfected patients with liver disease (G3). As expected, uncontrolled HIV replication (G4–G6) led to a decline in $CD4^+$ T-cell counts. The fact that HIV patients who have cleared HCV (G4) showed increased cytokine plasma values together with an inverted CD4/CD8 ratio confirms the direct effect of HIV on immune activation. However, in HIV/HCV subjects with lack of HIV control and liver damage (G6), it is difficult to attribute immune activation to the presence of HIV or to the liver disease progression related to HCV infection. It seems likely that the combination of both factors results in the high levels of chronic immune activation observed in these individuals.

We observed that both proinflammatory and immunoregulatory cytokine levels were increased in uncontrolled HIV replication. It was suggested that an increased IL-10 production may have a protective role against HIV disease progression by diminishing the chronic immune activation, a major factor in HIV pathogenesis [33]. With this is mind, it could be possible that the increased immunoregulatory cytokine levels act as a compensatory mechanism to counteract the increased production of proinflammatory cytokines seen in untreated HIV infection, as an attempt to reduce the immune activation.

While, in HCV infection, there is controversial information regarding increased Th1 or Th2 cytokine levels, our results showed a preferential proinflammatory cytokine profile related to HCV presence. However, an increase of immunoregulatory cytokine levels was also observed in the presence of HCV-mediated liver pathology. TGF-β is a known fibrogenic cytokine that through the activation of hepatic stellate cells increase extracellular matrix production leading to hepatic fibrosis [34]. In contrast, IL-10 appears to act as an antifibrotic cytokine [35]. Therefore, in the context of HCV-associated liver disease, it could be possible that the increased IL-10 levels arise to suppress the fibrogenic effects caused by both proinflammatory and TGF-β cytokines. Nevertheless, this strategy would not seem sufficient to prevent hepatic damage.

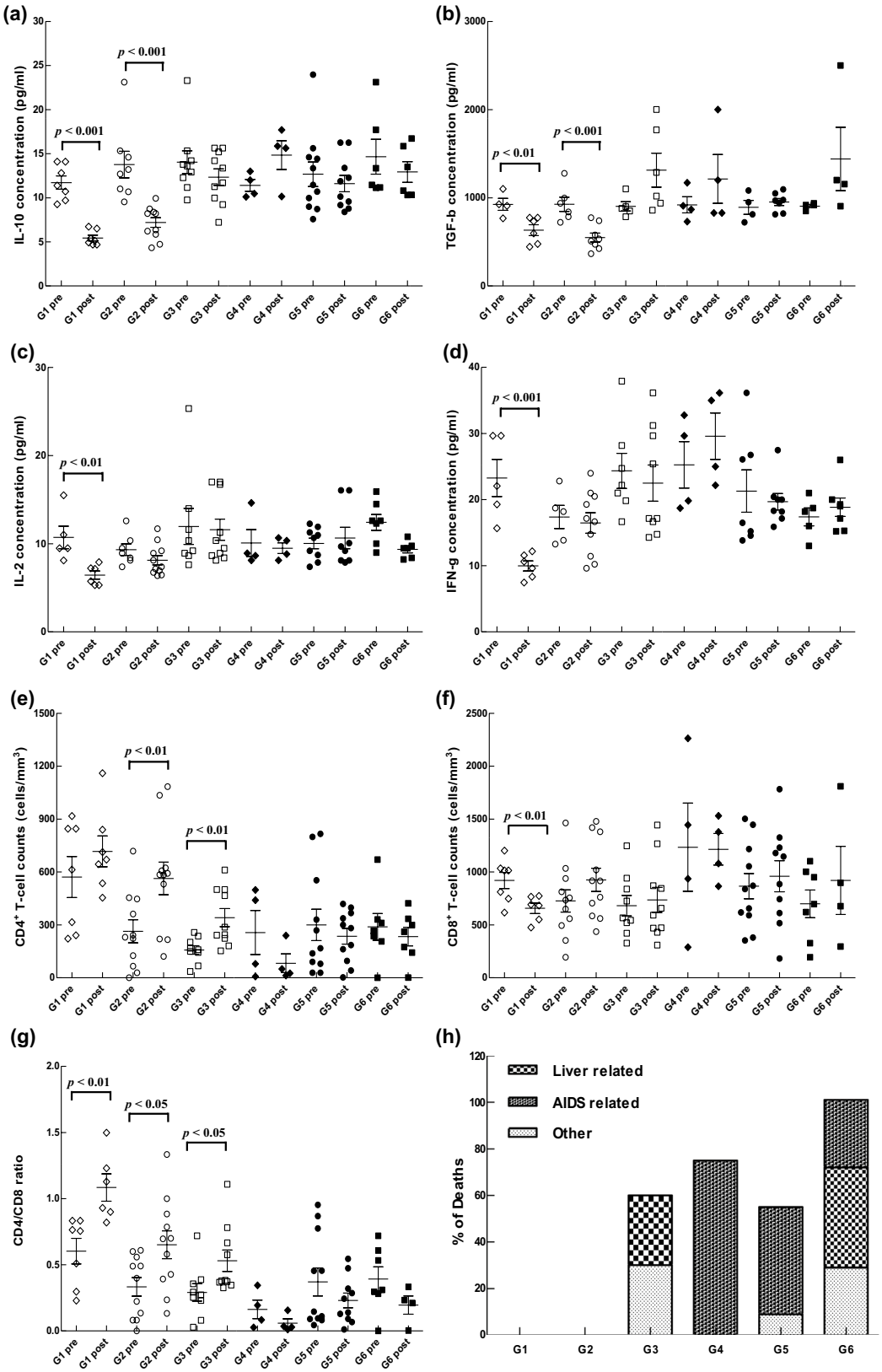
To investigate the importance of ART on immunological reconstitution, we compared the levels of the different immune parameters measured before and after ART onset. We observed that only subjects with controlled HIV infection and cleared HCV showed a significant reduction of immune activation levels, achieving immunological parameters values comparable to those of the control group.

Finally, we investigated disease evolution among HIV/HCV individuals by analyzing the percentages and causes of mortality in each group. Most of the patients who died were untreated HIV subjects (G4–G6), being AIDS-related complications the most frequent cause of decease. However, among patients with liver disease, 50% of deaths within the group on stable ART (G3) and 43% of deaths within the group without ART (G6) were related to liver disease progression. We also noted that individuals within G1, which presented normal $CD4^+$ T-cell counts at pre-ART stages, developed a better clinical outcome. This is in agreement with previously published data that indicates a significantly enhanced clinical outcome for individuals starting ART at CD4 counts > 500 cells/mm^3 [36]. This highlights the importance of the implementation of both HIV and HCV therapies, not only to reduce immune activation but also the mortality rates.

One of the limitations of our study is the small number of patients within each study group. The need of available information in clinical records for a long-term follow-up together with the requirement of fulfilling the eligibility criteria for each group limited the number of patients that could be involved in the study. Therefore, only 50 patients who fulfilled these requirements and allowed us to study the individual contribution of HIV viral load, HCV status, and liver disease on immune activation and reconstitution in HIV/HCV patients could be included in the current work. Besides, subjects without control of HIV replication are less likely to achieve HCV clearance. This explains the small number of patients included in the group 4 (G4, $n = 4$) as compared with the other groups.

In summary, in the present study, we analyzed the presence of underlying immune activation in a cohort of HIV/HCV subjects with different disease outcomes. Since uncontrolled HIV replication has been proved to be a potent stimulus to drive chronic immune activation, we particularly focus on the presence of HCV and its liver-related disease as putative driving forces that may account for immune activation. Our results depict that, despite successful HIV suppression by ART, ongoing immune activation remains in HIV/HCV-coinfected patients. Both HCV presence and its associated liver pathology act as pathogenic factors that contribute to immune activation leading to an inappropriate immune reconstitution.

As the current HCV treatments with direct-acting antiviral (DAA) drugs are expensive, most countries (including Argentina) have been established a list of patients with priority to be treat with DAA that includes those with cirrhosis or advanced fibrosis, coinfected with HIV


(a)
IL-10 concentration (pg/ml)
p < 0.001
p < 0.001
(b)
TGF-b concentration (pg/ml)
p < 0.01
p < 0.001
(c)
IL-2 concentration (pg/ml)
p < 0.01
(d)
IFN-g concentration (pg/ml)
p < 0.001
(e)
CD4+ T-cell counts (cells/mm3)
p < 0.01
p < 0.01
(f)
CD8+ T-cell counts (cells/mm3)
p < 0.01
(g)
CD4/CD8 ratio
p < 0.01
p < 0.05
p < 0.05
G1 pre
G1 post
G2 pre
G2 post
G3 pre
G3 post
G4 pre
G4 post
G5 pre
G5 post
G6 pre
G6 post
(h)
% of Deaths
Liver related
AIDS related
Other
G1
G2
G3
G4
G5
G6

◂**Fig. 3** State of immune activation before (pre) and after (post) the implementation of antiretroviral treatment (ART). Levels of: **a** IL-10, **b** TGF-β, **c** IL-2, **d** IFN-γ, **e** $CD4^+$ T cells, **f** $CD8^+$ T cells, and **g** CD4/CD8 were measured at pre- and post-ART stages in each patient group. **h** Rates and causes of death among the groups of HIV/HCV coinfected patients. *p* values were calculated with unpaired *t* test or Mann–Whitney test. Means ± SEM are indicated. G1: HIV suppressed subjects with cleared HCV, G2: HIV suppressed subjects with chronic HCV infection, G3: HIV suppressed subjects with chronic HCV infection and hepatic damage, G4: HIV subjects with cleared HCV, G5: HIV subjects with chronic HCV infection, and G6: HIV subjects with chronic HCV infection and liver damage

among others. The decision to include HIV/HCV coinfected patients in the priority list of treatment was made considering the impact of HIV on liver disease progression. In the present work, we observed that HCV presence and its associated liver pathology lead to an impaired immune reconstitution and clinical status in HIV-coinfected patients. Our results and the fact that, in the ART era, liver-related death has emerged as the main cause of mortality in HIV/HCV patients [37] support the priority of HCV treatment with DAA in HIV/HCV patients, not only because of the effect of HIV presence on liver damage, but also because of the impact of HCV coinfection upon the immune system reconstitution and disease progression. It would be interesting to investigate if the alterations of the immune system detected in the cohort of HIV/HCV patients studied could also be observed in HCV monoinfected patients with and without liver damage. If so, this may help to extend the list of patients who should receive treatment with DAA, including HCV patients without advanced liver disease, to achieve, besides the HCV elimination and prevention of liver disease progression, a reversion of the immune system alterations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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