Hepatitis C virus drives increased type I interferon-associated impairments associated with fibrosis severity in antiretroviral treatment-treated HIV-1-hepatitis C virus-coinfected individuals

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Background: Viral coinfections might contribute to the increased immune activation and inflammation that persist in antiretroviral treatment (ART)-treated HIV-1 patients. We investigated whether the hepatitis C virus (HCV) coinfection contributes to such alterations by impairing the plasmacytoid dendritic cell (pDC) IFNα/TLR7 pathway in a highly homogeneous group of ART-treated HIV-1–HCV-coinfected patients.

Methods: Twenty-nine HIV-1-infected patients with fully suppressive ART were included, 15 of whom being HCV-coinfected with mild-to-moderate fibrosis and matched for their HIV-1 disease, and 13 control healthy donors. Cellular activation, plasma levels of inflammatory cytokines and pDC transcriptome associated with IFNα/TLR7 pathway were characterized.

Results: Higher plasma levels of type-I interferon (IFN)-associated cytokines [interferon gamma-induced protein 10 (IP-10), MIP-1β, IL-8 and IFN-inducible T-cell alpha chemoattractant) were observed in HIV-1–HCV-coinfected than in HIV-1-monoinfected patients ($P = 0.0007$, 0.028, 0.028 and 0.035, respectively). The pDCs and T cells displayed a more exhausted (LAG-3+ and CD57+, respectively) phenotype. The pDC IFNα pathway (defined by phosphorylated STAT1 expression) was constitutively activated in all patients, irrespective of HCV coinfection. Expression of interferon-stimulated genes (ISGs) *EI2AK2*, *ISG15*, *Mx1* and *IFI44* was increased in pDCs from HIV-1–HCV-coinfected individuals and was correlated with fibrosis score (Fibroscan, www.echosens.com, Paris, France and aspartate-aminotransferase/platelet-ratio index score, $P = 0.026$ and 0.019, respectively). Plasma levels of IP-10, STAT1 expression in pDCs and *Mx1* mRNA levels in pDCs decreased after interferon-free anti-HCV treatment.

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Conclusion: HCV replication appears to drive increases in type-I IFN-associated inflammation and ISGs expression in pDCs, in association with fibrosis severity in ART-treated HIV-1-infected patients with mild-to-moderate fibrosis. Preliminary results indicate reduction of these alterations with earlier interferon-free anti-HCV treatment in those patients.

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Keywords: hepatitis C virus, HIV, interferon α, inflammation, interferon-stimulated genes, IP-10, plasmacytoid dendritic cell

Introduction

Chronic immune activation and inflammation play a deleterious role during infection by the HIV type 1 (HIV-1) and persist despite fully suppressive antiretroviral treatment (ART), increasing the risk of serious non-AIDS-related morbidity and mortality in treated patients [1]. Although these alterations can be directly and indirectly enhanced by persistent production of type-I interferons (IFN) immune deregulation, loss of lymphoid tissue integrity and microbial translocation linked to HIV-1 replication [2,3], virus coinfections might also play a key role. Indeed the cytomegalovirus (CMV) coinfection has been shown to increase the immune activation caused by HIV-1, whereas anti-CMV treatment can reduce such activation [1]. Coinfection with hepatitis C virus (HCV) might play a similar role either directly or indirectly, by exposing to accelerated hepatic fibrosis progression, higher rates of liver failure and death [4], and has been associated with a 35% increased risk of overall mortality compared with HIV-1 monoinfection in the post-ART era [5]. Whether such increased morbidity stems directly from HCV replication or results from collateral damages from long-standing inflammation induced by HCV coinfection remains unclear [6]. In addition, a controversy persists as to whether the HCV coinfection increases immune activation linked to HIV-1 as compared with HIV-1 monoinfection [7–10].

Both HIV-1 and HCV single-stranded RNAs activate plasmacytoid dendritic cells (pDCs) via TLR7 [11–13] and can impair pDC-associated IFNα/TLR7 pathways [14,15]. IFNα is mainly produced by pDCs in response to endosomal TLR stimulation and is involved in several aspects of HIV-1 immune pathogenesis [2]. Contrasting with the loss of circulating pDCs during both HIV-1 and HCV monoinfections and coinfections [16–20], higher IFNα plasma levels have been reported in small numbers of coinfected versus HIV-1-monoinfected patients so far [21]. HCV is also known to induce strong interferon response/interferon-stimulated genes (ISGs) in the hepatic compartment [22–24], whereas preactivation of IFNα signaling in the liver, defined by higher expression of ISGs and phosphorylation and nuclear localization of the STAT1 transcription factor, has been associated with refractoriness to IFNα-based treatment [25]. However, systemic IFN induction or preactivation of IFN pathway is not as evident [25–28], and many confounding factors can bias the results such as sex, multiple HCV genotypes or heterogeneous degrees of liver fibrosis. Therefore, although alterations in the pDC IFNα/TLR7 pathway may shape the immune activation and inflammation in HIV-1–HCV coinfection, conclusive data are lacking. In addition, even if HCV direct-acting antiviral (DAA) agents offer tremendous progresses in the treatment of HCV, their indication is still limited to advanced HCV infection [29], and there is a need to better evaluate whether the early stages of chronic HCV infection can aggravate the immune activation and inflammation associated with HIV-1 and have deleterious consequences for comorbidities and HCV pathogenesis.

Understanding whether HCV coinfection increases the systemic immune activation and/or inflammation in HIV-1-infected patients with suppressive ART should help elucidate challenging issues for treating HIV-1 patients with HCV coinfection. To test such hypothesis, we designed a highly homogeneous study that includes only HIV-1-infected patients on suppressive ART, half of them being HCV coinfected and matched for HIV-1 disease and personal characteristics with the HCV-negative HIV-1-monoinfected patients. We investigated whether the HCV coinfection caused by a single genotype and moderate fibrosis drives an increase in parameters of inflammation and cellular immune activation in HIV-1-infected patients under suppressive ART by analyzing the pDC IFNα/TLR7 pathway and expression of activation markers and ISGs.

Material and methods

Study design, patients and samples

We recruited 29 ART-treated chronically HIV-1-infected male adults followed at Pitié-Salpêtrière and Saint-Antoine hospitals, with plasma HIV-1 RNA levels less than 50 copies/ml and $CD4^+$ cell counts at least 350 cells/μl for at least 6 months. Half had to be coinfected by a genotype-1 HCV, naive for HCV treatment, with detectable HCV

loads and matched with the HCV-negative patients for age (20–40 or 40–60 years old), ethnicity, $CD4^+$ nadir, known duration of HIV-1 infection and ART. Liver fibrosis was assessed using transient elastography (Fibroscan; www.e-chosens.com, Paris, France) and classified using the METAVIR score (F0–F2: mild-to-moderate fibrosis, F3–F4: severe fibrosis to cirrhosis) [30], FIB-4 score defined by [age (year) × aspartate aminotransferase (AST) (IU/l)]/[(platelet count (PLT) (10^9/l)) × (alanine transaminase (ALT) (IU/l))$^{1/2}$] and the aspartate aminotransferase/platelet-ratio index (APRI) score, defined by [AST (IU/l) × 100]/[upper normal limit of AST (IU/l) × platelet count (10^9/l))] were calculated for all patients [31]. Additional samples were obtained from four HCV–HIV-1-coinfected patients 12 weeks after a 12-week long interferon-free anti-HCV therapy. Patient characteristics are presented in Table 1. Five additional HIV-1–HCV-coinfected patients with severe fibrosis were recruited on similar criteria except for previous HCV treatment, which had to be stopped for more than 6 months. Exclusion criteria were autoimmune or inflammatory diseases, hepatitis-B coinfection, active intravenous drug use and alcohol abuse. The study was approved by the local IRB (CPP-IdF6). We also included 13 normal samples from blood bank healthy donors. All individuals provided a written signed informed consent.

Blood collected on sodium heparin was processed within 5 h maximum. Plasma was stored at −80 °C. PBMCs were isolated by Ficoll density gradient and either used directly or cryopreserved in liquid nitrogen.

Plasma cytokines levels

Plasma samples were run on the ProCarta Human Ultrasensitive 9-plex kit (IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL12p70, IL-17A and TNFα) (eBiosciences, San Diego, California, USA), the simplex TGF-β and customized 7-plex (IFNα, IL-8, IP-10, TNFα, I-TAC, TRAIL, sCD40L and MIP-1β) (eBiosciences). All IFNα subtypes were measured using the human IFNα ELISAPRO kit (Mabtech, Sweden). Lower limits of quantification are indicated in Table S1, http://links.lww.com/QAD/B62.

Flow cytometry analysis

Whole blood samples (100 μl) were stained for T-cell markers with anti-CD3-BV711, anti-CD4-BV605, anti-HLA-DR-antigen-presenting cells (APC)-Cy7, anti-CD25-BV421, anti-CD38-PE-Cy7, anti-CD57-PE, anti-CD69-FITC (BD Biosciences, San Jose, California, USA), anti-CD8 Alexa-Fluor (AF)700 (Beckman-Coulter, Fullerton, California, USA) and for APC markers (500 μl), using anti-CD14-BUV395, anti-CD3/CD56/CD19-AF700, anti-CD11c-APC, anti-CD123-PE-Cy5, anti-HLA-DR-BV421, anti-CD16-APC-Cy7, anti-CD163-PE-CF594, anti-CD40-PE, anti-CD80-PE-Cy7, anti-CD86-FITC (BD Biosciences) and anti-CCR7-BV711 (eBiosciences), followed by lysis and fixation (BD Lysis-Buffer; BD Biosciences, San Jose, CA). For T-cell activation, cells were subsequently permeabilized (Perm-Buffer-B; Invitrogen, Carlsbad, California, USA) and stained with anti-Ki67-AF647 (BD Biosciences). Absolute quantification of pDCs, $CD4^+$ and $CD8^+$ T cells was performed using Trucount tubes (BD Biosciences).

For phosphoproteins measurement in pDCs, fresh PBMCs were stained using anti-CD3/19/56-BV421, anti-CD11c-PE, anti-CD14-PE-Cy7, CD123-PE-Cy5 (BD Biosciences), anti-HLA-DR-eFluor605NC (eBiosciences), paraformaldehyde-fixed and permeabilized by methanol and stained with anti-pIRF7-AF488 and anti-pSTAT1-AF647 (BD Biosciences).

For analysis of Mx1 mRNA and STAT1 protein expression, PBMCs stained by anti-CD3-BV711, anti-CD19/56/11c/14-AF700, anti-HLA-DR-BV421, anti-CD8-PE-Cy7, anti-CD57-PE, anti-CD123-PE-Cy5, anti-CD4-BV605, anti-CD38-PE-CF594 and anti-PD-1-BV786 (BD Biosciences) were subjected to the QuantiGene PrimeFlow RNA assay (eBiosciences) with type6-*Mx1* and type4-*PKR* probes, bacterial *DapB* probe as control (eBiosciences) and anti-STAT1-AF647 (BD Biosciences).

All samples were acquired on BD Biosciences Fortessa within 3 h of staining and analyzed using the FlowJo software (Treestar, Ashland, Oregon, USA).

Transcriptome analysis

Freshly thawed PBMCs were stained by anti-CD3-PerCP-Cy5.5, anti-CD19/56/11c/14-AF700, anti-HLA-DR-APC-Cy7, anti-CD8-PE-Cy7, anti-CD57-PE, anti-CD123-PE-Cy5 (BD Biosciences), anti-CD303-APC (Miltenyi-Biotec, Bergisch Gladbach, Germany), anti-LAG-3-FITC (R&D Systems, Minneapolis, Minnesota, USA) and the viability dye Zombie-Red (eBioscience). pDCs defined as live CD3negCD56-negCD19negCD14negCD11cneg HLA-DRposCD123-brightCD303pos were sorted using a FACS Aria Fusion (BD Biosciences). One hundred live pDCs were sorted into a 96-well PCR plate preloaded with lysis-buffer (Life Technologies) and immediately frozen. Sequences of primer pair oligos (DeltaGene; Fluidigm, South San Francisco, California, USA) were validated in-house (Table S2, http://links.lww.com/QAD/B62). cDNA prepared using SuperScript Enzyme-Mix (Invitrogen) and *T4*-Gene 32-Protein (New-England Biolabs, Ipswich, Massachusetts, USA) was preamplified (PreAmp Master-Mix; Fluidigm) treated with exonuclease I (New-England Biolabs). Sample premix (Fast EvaGreen-Supermix; Bio-Rad, Hercules, California, USA) and assay premix were transferred to the primed GE-Dynamic-Array 96.96 (Fluidigm). qPCR of the 83 preamplified prediluted cDNA samples, a no template control and internal control from FLU-stimulated Gen2.2 cells was conducted on the

Table 1. Study patients' characteristics.

Groups	HIV-1-monoinfected individuals, $n=14$	HIV-1–HCV-coinfected individuals, $n=15$	Healthy controls, $n=13$	*P* value
General characteristics				
Sex	Male	Male	Male	
Age (year) (range)	51.73 (40–59)	47 (36.5–54)	38.3 (23–51)	0.04
Ethnicity			NA	0.32[a]
White [number (%)]	13 (93%)	11 (73%)		
African [number (%)]	1 (7%)	1 (6.6%)		
Maghrebian [number (%)]	0 (0%)	1 (6.6%)		
West Indian [number (%)]	0 (0%)	1 (6.6%)		
Unknown [number (%)]	0 (0%)	1 (6.6%)		
BMI $\geq 25\,kg/m^2$	5 (35.7%)	5 (33%)	NA	1[a]
History of IDUs			NA	
Active [nombre (%)]	0 (0%)	0 (0%)		0.018[a]
Past [nombre (%)]	0 (0%)	5 (33%)		
No [nombre (%)]	14 (100%)	10 (66%)		
HIV-1 disease characteristics				
HIV-1 plasma VL (copies/ml)	<20	<20 (except 1 VL = 54)	NA	–
$CD4^+$ absolute median (cells/μl) (range)[b]	605 (380–1033)	716.6 (400–1478)	NA	0.18
$CD4^+$ median % (range)[b]	34.3 (24–46)	32.8 (19–46)	NA	0.73
$CD8^+$ absolute median (cells/μl) (range)[b]	676 (442–1139)	922 (406–2262)	NA	0.21
$CD8^+$ median % (range)[b]	39.3 (25–56)	41.4 (26–63)	NA	0.68
Ratio $CD4^+/CD8^+$ median (range)[b]	0.76 (0.46–1.99)	1.03 (0.37–1.84)	NA	0.98
Nadir $CD4^+$				
<200 [number (%), range]	8 (57%), 1–195	8 (53%), 3–171		–
200–350 [number (%)]	5 (35.7%)	5 (33%)		–
>350 [number (%)]	1 (7%)	2 (13%)		–
Estimated duration of HIV-1 infection			NA	
Years (range)	18.3 (6–27)	18.8 (4–28)		0.84
>10 years (%)	13 (93%)	14 (93%)		–
ARV therapy			NA	
Years of ART (range)	15.9 (3–26)	12.2 (3–23)		0.19
Years between diagnostic and HAART (range)	2.7 (0–9)	6.6 (0–21)	NA	0.32
ART regimen			NA	
PI-based regimen (%)	9 (64%)	8 (53%)		0.71[a]
Current use of atazanavir (%)	1 (7%)	3 (21%)		0.59
Former use of maraviroc (%)	1 (7%)	1 (7%)		1
HCV disease characteristics				
Estimated duration of HCV infection				
Years (range)	NA	12 (2–23)	NA	
>10 years (%)	NA	9 (60%)	NA	
HCV treatment	NA		NA	
Naive [number (%)]		15 (100%)		
Previous interferon-based treatment [number (%)]		0 (0%)		
Median HCV VL (IU/ml)	NA	2047 170 (207 026–5842 054)	NA	–
HCV genotype				
% Genotype 1a	NA	10 (66%)	NA	–
% Genotype 1b	NA	5 (33%)	NA	–
% Unknown	NA	0 (0%)	NA	–
Order of infection		HIV-1 infection first		
Liver fibrosis/cirrhosis				
% F0–F1 [number (%)]	NA	14 (93%)	NA	
% F2 [number (%)]	NA	1 (7%)[c]	NA	
% F3–F4 [number (%)]	NA	0 (0%)	NA	
Patients with Fibroscan score available within 6 months [number (%)]	NA	11 (73%)	NA	
Fibroscan score (range)[d]	NA	6.5 (4.8–12.2)	NA	
ALT (IU/l) (range)[b]	30 (8–105)	55 (39–92)	NA	0.001
Patients with elevated ALT (%)[e]	1 (7%)	1 (7%)	NA	
AST (IU/l) (range)[b]	29.8 (21–49)	42 (28–61)	NA	0.004
Patients with elevated AST (%)[f]	0 (0%)	1 (7%)	NA	
APRI score[b]	0.37 (0.27–0.53)	0.54 (0.24–0.96)	NA	0.033
FIB-4 score[b]	1.49 (0.99–2.24)	1.36 (0.82–2.99)	NA	0.29
IL28B genotype (rs12979860)		NA	NA	
% Genotype C/C		4 (26.7%)		
% Genotype C/T		3 (20%)		
% Genotype T/T		2 (13.3%)		
% Unknown		6 (40%)		

ALT, alanine aminotransferase; APRI, aspartate aminotransferase/platelet-ratio index; ART, antiretroviral treatment; AST, aspartate aminotransferase; HCV, hepatitis C virus; NA, not available, not applicable; VL, viral load.
[a]Fisher exact.
[b]Sample collection within 1 month of the inclusion (sample from 1 HCV–HIV-1-coinfected individuals was collected 2.5 months before inclusion).
[c]Inconsistent diagnostics between fibroscan and fibrotest.
[d]For patients with fibroscore available within 6 months of the inclusion.
[e]Patients with more than two times the upper normal limit (normal ranges for ALT: 11–40 IU/l).
[f]Patients with more than two times the upper normal limit (normal ranges for AST: 10–30 IU/l). Mann–Whitney.

BioMark HD system (Fluidigm). Invalid reactions, determined using the Real-time PCR-Analysis Software (Fluidigm), were treated as missing data. Raw-Cq values were processed with Genomics-Suite (Partek, Saint-Louis, Missouri, USA). Outliers were excluded on the basis of principal component analysis (PCA). Three coinfected and one monoinfected individual's datapoints and two datapoints from coinfected individuals with severe fibrosis were removed from further analysis. Comparisons were analyzed on 17 coinfected (three with severe fibrosis), 14 monoinfected individuals and nine healthy donors. The reference gene *PTMA* was subtracted to target gene *Cq*. The mean between biological duplicates was calculated. Differentially expressed genes (DEGs) comparing patients groups with healthy controls were selected at more than two-fold difference and *P* value with false discovery rate (FDR) less than 0.05.

RNA was extracted from 100 000 PBMCs [RNeasy Mini-kit (Qiagen, Venlo, The Netherlands), DNase-treated and the prepared cDNA (Eurogentec, Liège, Belgium)] was preamplified and treated as above with 12 primers (*STAT1*, *Mx1*, *EI2AK2*, *ISG15*, *IFI44*, *IFNAR1*, *TP53*, *NLRP3*, *PTMA*, *GNAS*, *IFIH1 and PRDM1*). qPCR was performed on FLEXsix (Fluidigm). Data analysis was performed as above.

Statistical analysis

Comparison between two HIV-1 groups and linear regression were calculated with the Wilcoxon rank (Mann–Whitney) and Spearman tests, respectively. Transcriptomes were analyzed using Partek Genomics-Suite as above. Normality tests (Kolmogorov–Smirnov, D'Agostino&Pearson omnibus and Shapiro–Wilk) were performed on the data measured in HCV + HIV+ patients (MX-1 levels in pDCs, MFI STAT1 in pDCs and IP-10 plasma levels). Comparison between baseline and post-treatment was calculated with paired Wilcoxon Rank tests or paired *t* tests based on the normality tests' results. Correction for multiple tests was done according to Benjamini–Hochberg [42].

Results

Patient characteristics

We enrolled 29 HIV-1-infected patients, including 14 HIV-1-monoinfected and 15 matched HCV-coinfected patients, and 13 healthy donors as controls. Patients' characteristics are shown in Table 1. All 29 HIV-1-infected patients were on suppressive ART with $CD4^+$ T-cell count $\geq$ 350 cells/μl and HIV-1 viral load below 20 copies/ml, except for a blip in one patient. HIV-1 patient subgroups did not differ for HIV-1 or ART duration, $CD4^+$ T-cell nadir. All coinfected patients had a genotype-1 HCV with median 2047 170 IU/ml HCV load. Due to the exclusion of any previous anti-HCV treatment, fibrosis levels were mild (F0–F1) except for one patient with F2 score. The alanine aminotransferase (ALT) and AST values were higher in coinfected patients than in monoinfected ($P = 0.001$ and 0.0037, respectively) with higher APRI scores ($P = 0.033$).

Hepatitis C virus drives a mild systemic inflammation and exhaustion but not activation of peripheral blood plasmacytoid dendritic cells and T cells in antiretroviral treatment-treated HIV-1-coinfected patients

Plasma levels of IP-10, IL-8, MIP-1β and ITAC were significantly higher in coinfected than in HIV-1-

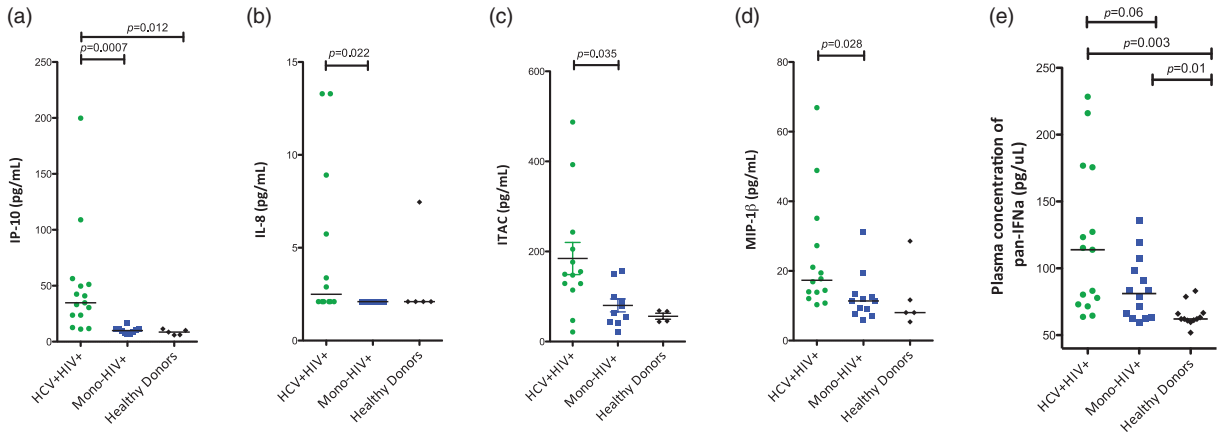

Fig. 1. Comparison of the plasma levels of inflammatory markers between hepatitis C virus–HIV-1 coinfected, HIV-1-monoinfected treated individuals and healthy controls. Plasma levels of IP-10 (CXCL-10) (a), IL-8 (b), ITAC (CXCL11) (c) and MIP-1β (d) were measured by Luminex in 15 hepatitis C virus–HIV-1-coinfected, 12 HIV-1-monoinfected treated individuals and five healthy donors. Plasma levels of panIFNα (all subtypes) (e) were measured by ELISA in 15 hepatitis C virus–HIV-1-coinfected, 12 HIV-1-monoinfected treated individuals and 12 healthy donors for panIFNα. Comparison between groups was performed by two-tailed Mann–Whitney *t* test. Horizontal bars show median. ITAC, interferon-inducible T-cell alpha chemoattractant; IL, interleukin; IP-10, interferon gamma-induced protein-10.

monoinfected patients (Fig. 1a–d, Table S1, http://links.lww.com/QAD/B62) with a trend toward higher IFNα levels in coinfected than in monoinfected individuals ($P=0.06$, Fig. 1e, Table S1, http://links.lww.com/QAD/B62). IP-10 in coinfected and IFNα levels in all HIV-1-infected individuals were significantly higher than in healthy donors (Fig. 1, Table S1, http://links.lww.com/QAD/B62). In addition to IL-1β, IL12p70, IL-2, IL-17A and IL-4 were not detected in any samples. These data show that HCV-1 coinfection with mild liver fibrosis drives only a mild increase in type-I IFN-associated inflammation in HIV-1-infected individuals under suppressive ART.

Levels of CD40, CD86, CCR7 and/or CD163 were then analyzed on pDCs, conventional DCs or monocytes subsets. None were influenced either by the HIV-1 or by the HCV status (Figs. S1 and S2A and B, http://links.lww.com/QAD/B62). No CD40 upregulation was observed in any subsets (Figs. S1A and S2A, http://links.lww.com/QAD/B62), and no CD86 upregulation was observed in pDCs (Fig. S2A, http://links.lww.com/QAD/B62). Nevertheless, a trend toward higher CD163 expression was observed on CD14++CD16−mono-CD16−monocytes in coinfected compared with mono-infected individuals (Fig. S1C, http://links.lww.com/QAD/B62, $P=0.07$). The expression of LAG-3, an exhaustion marker expressed by a pDC subset [32,33], although low, was significantly higher in pDCs from coinfected than from monoinfected individuals (Fig. S2C and D, http://links.lww.com/QAD/B62, $P=0.04$).

We next evaluated the T-cell activation levels. Although the CD25 and CD69 expression did not vary across patient groups (Fig. S1F, http://links.lww.com/QAD/B62), the CD38 and HLADR coexpression was significantly higher in HIV-1-infected individuals compared with healthy donors ($P=0.62$ and 0.53 for $CD4^+$ and $CD8^+$ T cells, respectively), irrespective of the HCV coinfection ($P=0.048$ and 0.016 for coinfected and monoinfected individuals, respectively), as well as the CD38 expression alone (Figs. S1G and S3, http://links.lww.com/QAD/B62). In addition, proportions of $CD8^+$ T cells expressing the immune-senescent marker CD57 were higher in coinfected patients (Fig. S3, http://links.lww.com/QAD/B62, $P=0.014$). As expected, both $CD4^+$ and $CD8^+CD57^+$ T cells were significantly higher in the whole HIV-1-infected group than in healthy donors ($P=0.0055$ and 0.0001, respectively), and $CD8^+CD57^+$ percentages were negatively correlated to the $CD4^+/CD8^+$ ratios ($P=0.018$, $R^2=0.19$). Neither the $CD38^+HLADR^+$ nor $CD57^+CD8^+$ T cells were correlated to HCV loads ($P=0.12$, $R^2=0.18$; $P=0.12$, $R^2=0.012$, respectively) or to the pDC LAG-3 expression. Altogether, these results suggest that in ART-treated HIV-1 infection with minimal $CD4^+$ T-cell defects, HCV is not associated with cellular immune activation but may rather contribute to immune exhaustion.

Impairment of the IFNα pathway in plasmacytoid dendritic cells in fully suppressed HIV-1 infection

We then investigated the activation of the IFNα pathway in peripheral blood pDCs by quantifying ex-vivo levels of phosphorylated STAT1 (pSTAT1) and IRF7 (pIRF7) (Fig. 2a). No difference was observed between coinfected and monoinfected individuals. All HIV-1-infected individuals had significantly more pSTAT1 than healthy donors, irrespectively of HCV coinfection (Fig. 2b,

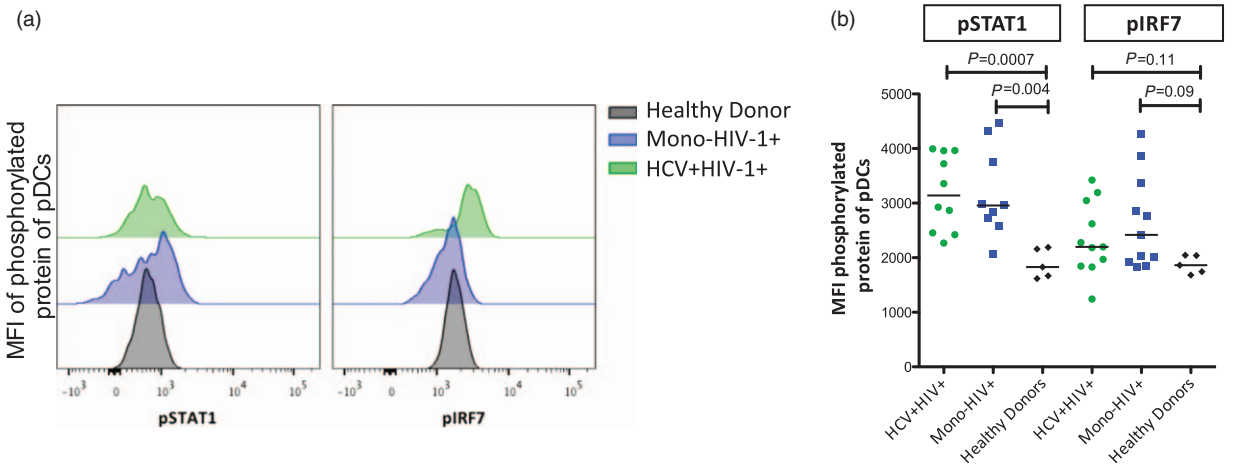

Fig. 2. Persistent activation of IFNα signaling in plasmacytoid dendritic cells (pDCs) in HIV-1-infected individuals despite suppressive antiretroviral treatment, irrespective of hepatitis C virus (HCV) status. (a) Representative flow cytometry histograms of the expression of phosphorylated STAT1 (left) and phosphorylated IRF7 (right) in pDCs. (b) Combined results for 15 HCV–HIV-1-coinfected individuals under suppressive antiretroviral treatment, 14 HIV-1-monoinfected individuals under suppressive ART and five healthy controls for the mean fluorescence intensity (MFI) of phosphorylated STAT1 and IRF7 in pDCs. Comparison between groups was performed by Mann–Whitney *t* test. Horizontal bars show median.

$P = 0.007$ and 0.004 for coinfected and monoinfected individuals, respectively) and displayed a trend toward higher pIRF7 compared with healthy donors (Fig. 2b, $P = 0.11$ and 0.09 for coinfected and monoinfected individuals, respectively). Altogether, these data suggest that despite suppressive ART, pDC IFNα pathway is persistently activated and may therefore contribute to immune dysfunction in well treated HIV-1-infected individuals.

Strong type-I interferons signature in plasmacytoid dendritic cells from hepatitis C virus–HIV-1-coinfected individuals under suppressive antiretroviral treatment

To further investigate the peripheral blood pDC IFNα pathway activation, we studied the transcription of 96 genes mainly related to type-I IFN/TLR7 pathway in sorted pDCs from 15 coinfected, 14 monoinfected individuals and nine healthy donors. A heat map of the results is shown in Fig. 3a. PCA allowed distinguishing healthy donors from HIV-1-infected individuals irrespective of their HCV status (Fig. 3b). There were eight and four DEGs in coinfected individuals with minimal fibrosis and in monoinfected individuals, respectively, with a *P* value with FDR of less than 0.05 and a two-fold or greater difference in relative expression between patient groups and healthy controls, as shown in the Venn diagram (Fig. 3c, Table S3, http://links.lww.com/QAD/B62). Four ISGs (*IFI44*, *EI2AK2/PKR*, *ISG15* and *Mx1*) and *STAT1* were upregulated in coinfected individuals only, compared with healthy donors, irrespective of fibrosis stage, allowing us to define an 'ISG score' (Fig. 3d). Other ISGs did not significantly differ between groups, including ADAR, CCR5, IP-10, IFI16, IFI27, IRF2, DDX58, IRF7, IRF8, TREX1, IRF1 and USP18 (data not shown). None of the aforementioned ISGs were increased in monoinfected individuals compared with healthy donors, whereas STAT1 mRNA was significantly upregulated in HIV-1-monoinfected individuals compared with healthy donors (Fig. 3d). These four ISGs upregulation appeared limited to coinfected pDCs as their expression did not differ in total PBMCs among any patients groups, as illustrated for *Mx1* and *EI2AK2* in Fig. S4, http://links.lww.com/QAD/B62. In addition, only one gene, the interferon induced with helicase C domain 1 (IFIH1) was at least two-fold upregulated in all HIV-1-infected patient groups compared with healthy donors (Fig. 3c). These overall results indicate that HCV-1 drives an ISG upregulation in pDCs from HIV-1–HCV coinfected patients.

Hepatitis C virus replication drives the activation of type-I interferons signaling

To explore whether hepatic fibrosis or HCV replication itself contributed to those type-I IFN-associated inflammation and transcriptomic signature observed in coinfected individuals, we obtained samples from four coinfected patients 12 weeks after completion of an interferon-free anti-HCV treatment. We measured *Mx1* mRNA expression and STAT1 protein expression in pDCs using intracellular PCR. Treatment-induced HCV clearance significantly reduced *Mx1* mRNA expression in pDCs (Fig. 4a and b, $P = 0.014$) down to post-treatment levels similar to those obtained for an irrelevant probe against bacterial DapB (Fig. 4a). A trend toward reduced levels of STAT1 in pDCs (Fig. 4c, $P = 0.069$) and reduced plasma levels of IP-10 (Fig. 4d, $P = 0.12$) were observed, but these differences did not reach significance due to the small number of samples available. Altogether, these data suggest that HCV replication may be driving increased activation of type-I IFN signaling at the systemic and pDC levels in HCV–HIV-1-coinfected individuals.

Immune impairments in hepatitis C virus–HIV-1 coinfection correlate with hepatitis C virus disease severity

Finally, we addressed whether such activation of type-I IFN signaling was linked to HCV disease severity. As our inclusion criteria lead us to enroll only coinfected patients with mild-to-moderate fibrosis, we recruited five additional coinfected individuals with more advanced fibrosis, as described in Table S4, http://links.lww.com/QAD/B62. The transcriptome of sorted pDCs from four of these individuals analyzed in parallel to the other patient groups showed 10 DEGs with three DEGs specifically regulated in this patient group (Fig. 3c, Table S3, http://links.lww.com/QAD/B62). The upregulation of the ISGs *IFI44*, *EI2AK2/PKR*, *ISG15* and *Mx1* was at least two-fold stronger in coinfected patients with severe fibrosis compared with patients with mild-to-moderate fibrosis (Fig. 3d). The 'ISG score' was correlated to HCV disease severity (Fig. 3e), as assessed by the Fibroscan score ($P = 0.026$, $R^2 = 0.35$), ALT levels ($P = 0.018$, $R^2 = 0.34$) and APRI score ($P = 0.019$, $R^2 = 0.4$) in coinfected individuals with mild-to-severe fibrosis but not to HCV viral load. This was not the case in monoinfected individuals (ALT levels: $P = 0.77$, $R^2 = 0.008$; APRI score: $P = 0.38$, $R^2 = 0.087$). The pDCs STAT1 expression was also correlated to ALT levels ($P = 0.0094$, $R^2 = 0.37$) and Fibroscan score ($P = 0.0012$, $R^2 = 0.60$) (Fig. 3). In contrast, plasma levels of IP-10, IL-8, MIP-1β and ITAC in coinfected individuals were independent of fibrosis stages.

To eliminate a confounding factor, we then observed that pDC absolute numbers were significantly lower in coinfected individuals with severe fibrosis than in individuals with minimal fibrosis or healthy donors as previously suggested [34,35]. Interestingly, the absolute counts of pDCs were not correlated to Fibroscan values but only to the $CD57^+CD8^+$ T-cell percentage (Fig. S5, http://links.lww.com/QAD/B62, $P = 0.026$, $R^2 = 0.35$). Altogether, these data suggest that increased activation of pDCs type-I IFN signaling is associated with HCV disease severity.

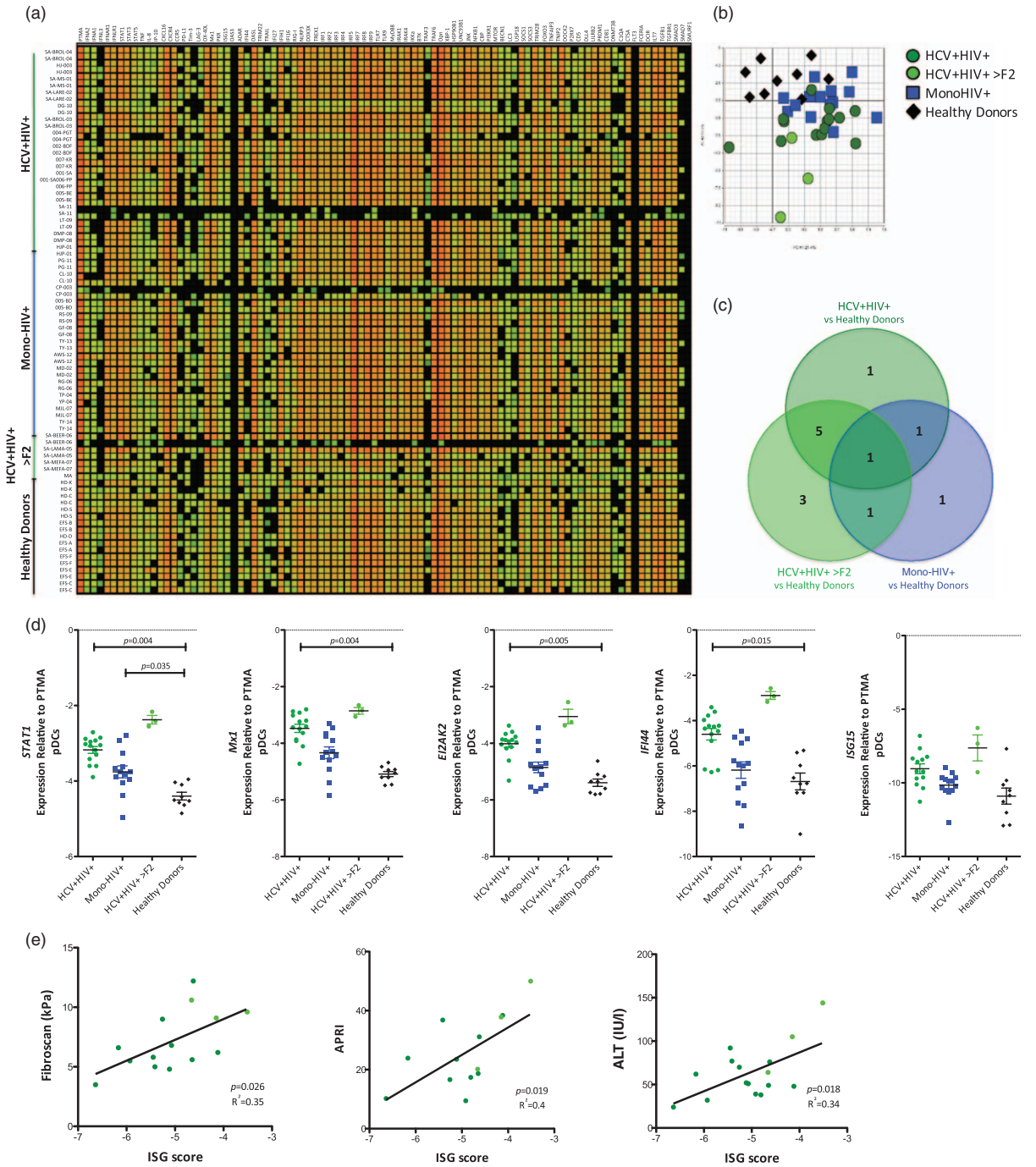

Fig. 3. Transcriptomic analysis of plasmacytoid dendritic cells (pDCs) from hepatitis C virus (HCV)–HIV-1-coinfected individuals with minimal-to-moderate fibrosis, severe fibrosis, HIV-1-monoinfected individuals and healthy donors. A total of 100 pDCs were sorted in biological duplicates for 15 HCV–HIV-1-coinfected, 14 HIV-1-monoinfected individuals, nine healthy controls and four HIV-1–HCV-coinfected individuals with severe fibrosis before being analyzed on the Fluidigm Biomark HD. (a) A heat map of the results is shown. (b) After exclusion of outliers and normalization to the housekeeping gene *PTMA*, mean between duplicates was calculated. Segregation between groups by principal component analysis is shown. (c) Differentially expressed genes (DEGs) in 14 HCV-HIV–1-infected patients with minimal-to-moderate fibrosis (HCV+HIV+), 14 HIV-1-monoinfected patients (MonoHIV+) and in four hepatitis C virus-HIV-1-infected patients severe fibrosis HCV+HIV+>F2), with a *P* value with false discovery rate of less than 0.05 and two-fold or greater difference in relative expression to nine healthy controls. (d) The relative expression of *IFI44*, *Mx1*, *ISG15*, *EI2AK2* and *STAT1* in pDCs is compared between the groups. Comparison was performed by Mann–Whitney *t* test with multiple tests correction (Benjamini et Hochberg, 1995). (e) Interferon-stimulated gene score, defined as the average of *IFI44*, *Mx1*, *ISG15* and *EI2AK2* expressions in pDCs, correlated to Fibroscan values, ALT levels and APRI score. Linear regression was calculated with Spearman rank-based correlation.

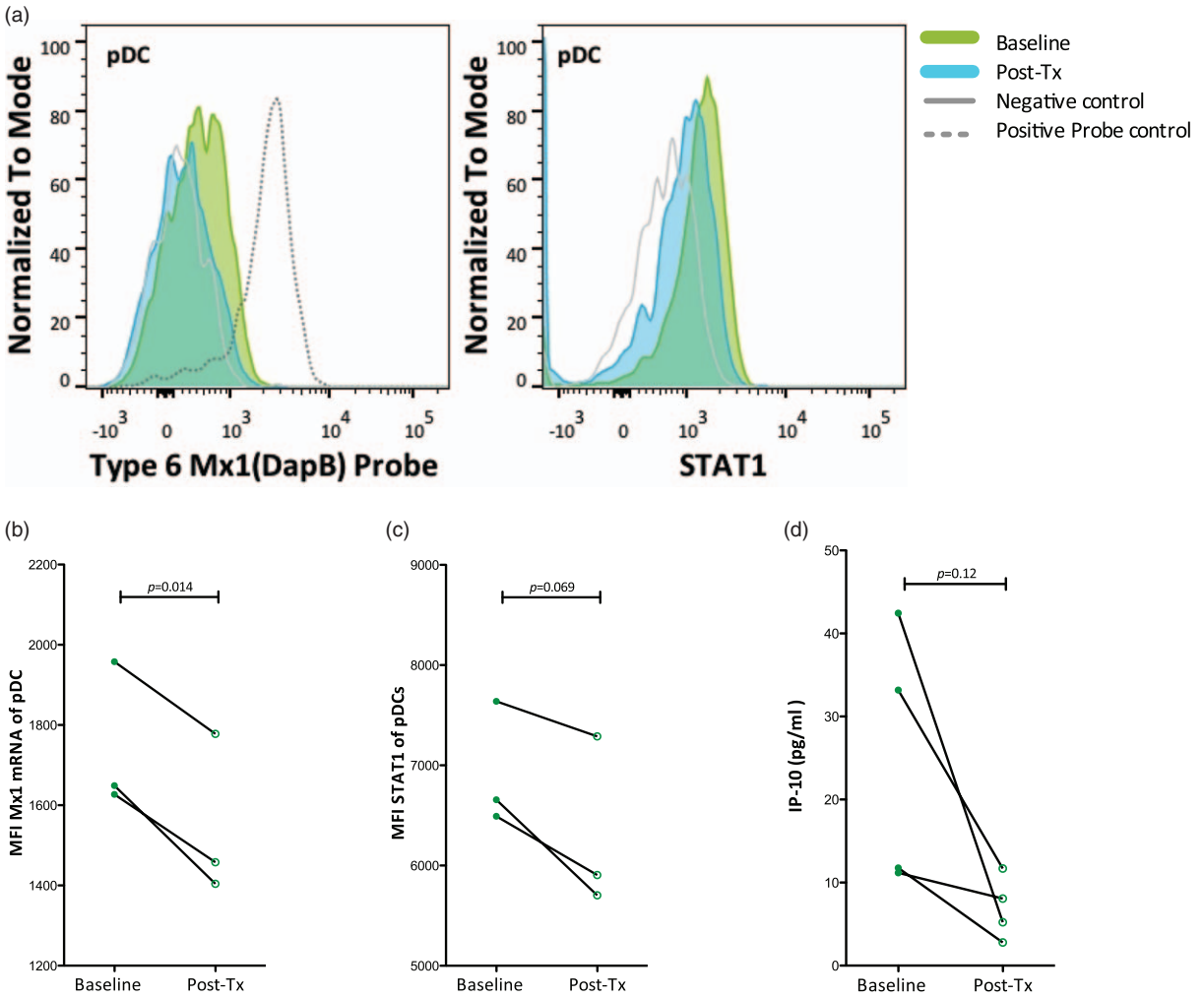

Fig. 4. Effect of treatment-induced hepatitis C virus (HCV) clearance on type-I interferon (IFN)-associated impairments in HCV–HIV-1-coinfected patients. Samples from four HCV–HIV-1-coinfected patients were collected at the time of inclusion (Baseline) and 12 weeks post-treatment completion (Post-Tx). (a–c) Mx1 mRNA and STAT1 protein expressions were evaluated in pDCs from three individuals using the PrimeFlow QuantiGene assay (eBiosciences). (a) Histograms overlay expression of type 6 probe directed against *Mx1* mRNA (left) and STAT1 protein expression (right) in pDCs from a representative hepatitis HCV–HIV-1-coinfected individual at baseline (green line) and 12 weeks post-treatment completion (Post-Tx) (blue line). Negative controls are shown in gray. Irrelevant type 6 probe against bacterial DapB mRNA was used as negative control for detection of *Mx1* mRNA. Fluorescent minus one (FMO) was used for controlling for STAT1 expression. Positive control for type 6 probe is shown in dotted gray line and was directed against RPL13A. (b) Mean fluorescence intensity (MFI) of *Mx1* mRNA in plasmacytoid dendritic cells is shown. (c) Mean fluorescence intensity of STAT1 protein in plasmacytoid dendritic cells is shown. (d) IP-10 plasma levels were measured by Luminex in four individuals. Comparison between baseline and post-treatment levels by paired *t* test (b and c) or paired Mann–Whitney *t* test (d) in agreement with normality tests performed on data generated from our HCV + HIV+ patients group.

Discussion

Our comprehensive study showed that a highly homogeneous untreated HCV coinfection with mild-to-moderate fibrosis induced a type-I IFN signature associated with increased inflammation in HIV-1-infected individuals. The most prominent markers of this signature were an increased expression of IP-10 and some ISGs, including *Mx1* in pDCs. The ISG expression score correlated with the levels of liver fibrosis, whereas both IP-10 plasma and *Mx1* mRNA levels in pDCs decreased after an interferon-free DAA treatment. Overall, this first extensive ex-vivo study of pDC numbers, activation, phosphorylation, transcription and function of key IFN pathway molecules without further in-vitro stimulation suggest that HCV genotype-1 replication heightens activation of type-I IFN signaling and aggravates immune alterations in HCV–HIV-1 coinfection, even in the context of minimal fibrosis.

Many controversies persist regarding the question of whether HCV coinfection increases T-cell activation or inflammation induced by HIV-1. Indeed, HCV is known to activate the liver type-I IFN pathway despite controversies at the systemic level [25,28,36], whereas chronic activation of the innate rather than adaptive immune system has been associated with increased morbimortality in ART-treated HIV-1 infection [37]. Our results add to the previously reported higher plasma levels of IP-10 and inflammatory cytokines in coinfected patients [38], by showing their association with increased exhaustion of peripheral blood pDCs and $CD8^+$ T cells but not with increased immune activation of T cells, DCs or monocytes. Of note, the $CD38^+HLADR^+CD8^+$ T-cell percentages were lower in all our study groups than in other studies, consistently with their profound HIV-1 suppression [9]. We also found blood pDC absolute counts to be correlated with $CD57^+CD8^+$ T-cell percentages in these ART-treated HCV–HIV-1-coinfected patients. Interestingly, both parameters had been correlated to ART duration irrespective of virus coinfection [39–41]. Indeed, in an effort to attenuate potential confounding factors in T-cell activation and inflammation, our study was restricted to patients under suppressive ART with normalized $CD4^+$ cell counts, coinfected by a single HCV genotype and with mild fibrosis, all matched for sex and for their $CD4^+/CD8^+$ ratio and $CD4^+$ nadir known to interfere with immune activation [41,42]. These highly homogeneous patient study groups differ from the various settings of co-HCV–HIV-1-infected patients with very different HIV-1 disease status or liver disease stages in whom previous studies had reported increased T-cell activation [7–9].

The typical ISG signature, with increased mRNA levels of *IFI44*, *EI2AK2/PKR*, *ISG15*, *Mx1* and of *STAT1* found in coinfected compared with monoinfected patients appeared to be limited to pDCs. Our study was not designed to study other minor populations. Therefore, we cannot exclude that another minor population display similar signature. These ISGs display antiviral activity against a broad spectrum of viruses including HCV and HIV-1 [43]. Our results also add *IFI44*, *EI2AK2/PKR* to previous results showing that increased ISG15 and MX1 expressions predict a sustained virological response to interferon-based anti-HCV treatment [44]. Though still preliminary, our results suggest *Mx1* mRNA expression decreases in pDCs after HCV clearance with antiviral treatment, thus complementing reports of IFI44 and/or ISG15 downregulation after interferon-free DAA treatment in HCV monoinfection [45,46]. HCV clearance is also associated with decreased IP-10 plasma levels that are partially upregulated by type-I IFN [47]. Moreover, strong ISGs expression in HCV–HIV-1 coinfection had suggested unresponsiveness to standard PegIFN + ribavirin therapy [48], but the liver and peripheral blood IFN pathway had not been compared after Peg-IFNα failure in HCV-infected patients to normal levels [25,26,28,49]. In the context of interferon-free DAA treatments, clinical practice guidelines no longer differentiate between HCV–HIV-1 coinfection and HCV monoinfection and recommend to treat only patients with significant fibrosis [50,51]. Excessive inflammation favors HCV-mediated liver damage and faster progression to fibrosis in HIV-1–HCV coinfection compared with either HIV-1 or HCV monoinfection [52,53]. To avoid heterogeneity and the inflammatory disorders linked to severe liver disease, we biased our recruitment strategy toward male sex, HCV genotype 1 and minimal fibrosis. Our results suggest that pDCs ISGs levels are linked to HCV disease severity and that HCV–HIV-1 coinfection with minimal fibrosis could benefit from an earlier interferon-free DAA treatment.

Altogether, this study shows that HCV–HIV-1 coinfection, even with minimal fibrosis, aggravates the HIV-related immune activation and inflammation, particularly the pDC type-I interferon signaling, in relation to HCV replication and provides a rationale for treating early HCV–HIV-1 coinfection even with mild fibrosis.

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Conflicts of interest

There are no conflicts of interest.

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