Decreased pro-inflammatory immune responses during recurrent acute HCV infections in HIV co-infected patients

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Abstract
Patients in high-risk groups continue to transmit the hepatitis C virus (HCV) and frequently experience reinfections. Since little is known regarding the immune response to HCV during reinfection, we compared primary and consecutive acute HCV infections in patients with an HIV infection, and focused on the cytokine bridging innate to adaptive immunity. We observed that the serum levels of IL-12p40, MIP-1β, MIG and IP-10 increased during primary acute HCV infection, but not during subsequent secondary acute reinfections. The weaker pro-inflammatory cytokine responses observed during HCV reinfections suggest more limited secondary acute immune responses, which may prevent damage to the infected liver.

INTRODUCTION
Hepatitis C virus (HCV) infections remain a global health problem, with 170 million people being chronically infected. HCV transmission is ongoing, especially among high-risk groups. These risk groups are people in developing countries with poor healthcare facilities, and people in developed countries who are injecting drug users or HIV-positive men who have sex with men (MSM) [1]. Potent antivirals directly targeting HCV have recently entered clinical practice with sustained viral response (SVR) rates well above 90 % [2], and in theory these could end the epidemic. Unfortunately, this seems not to be the case, because recent modelling studies have shown that the HCV epidemic amongst MSM continues to expand, partly due to continuing high-risk behaviour resulting in multiple infections [3].

Following infection with HCV, viral loads increase exponentially over the first few weeks of infection without overt symptoms in the majority of patients. In the infected liver, chemokines are pivotal, not only for the recruitment of innate immune cells, including NK cells, NKT cells, monocytes and plasmacytoid dendritic cells, but also for adaptive immune responses, including the augmentation of HCV-specific interferon (IFN)-γ and tumor necrosis factor (TNF) production by T cells. Their production is triggered by the infection of hepatocytes by HCV, and the activation of pattern-recognition receptors on resident liver cells and type I IFNs [4]. Four to six weeks after infection, there is an adequate response of the innate immune system and HCV RNA levels stabilize afterwards, mainly due to type I IFN-mediated responses, which direct the antiviral activity of NK cells through the upregulation of perforin and IFN-γ [5]. Simultaneously, HCV-specific CD4+ and CD8+ T-cell responses are detected, directed against multiple epitopes in the blood [6]. These responses generally persist longer in patients who spontaneously resolve their infection, but not in individuals who are unable to clear HCV [6, 7].

Little is known about the consequences of spontaneous HCV clearance for immunity and whether it affects immunological events during reinfection. Our current knowledge mainly relies on cross-sectional data during reinfection. The probability of spontaneous clearance depends on favourable IL28B rs12979860 CC gene polymorphisms, protective T-cell memory during reinfection and spontaneous clearance of the primary infection [8]. However, patients who spontaneously clear a primary infection remain susceptible to subsequent infections and can even become chronic [9, 10]. Longitudinal data are lacking and immune responses against HCV reinfection have not been carefully compared to responses against primary infection. Therefore, in this paper we compare the immune responses during primary
and consecutive acute HCV infections by studying plasma chemokines and the cytokine bridging innate to adaptive immunity.

RESULTS

Limited immune activation during chronic HIV mono-infection and chronic HIV/HCV co-infection
To establish the effect of HIV infection and/or HCV infection on cytokine and chemokine levels in plasma, we performed multiplex cytokine arrays for eight soluble proteins. The plasma levels for seven (IL-12p40, IP-10, MIG, MIP-1β, IL-6, IL-18 and IFN-γ) of the eight pro-inflammatory cytokines and chemokines tested were not elevated during chronic HIV (n=15) and chronic HCV/HIV co-infections (n=10) in comparison to healthy individuals (n=10). The baseline characteristics of the study subjects are presented in Table S1 (available in the online Supplementary Material). Only sIL-2R was significantly elevated during chronic viral (co-) infection (Fig. 1 and Table S2).

Increased cytokine/chemokine levels during primary acute HCV infection
We selected 15 HIV-infected patients with two consecutive HCV infections that were both diagnosed during the acute phase. Twelve patients were treated with cART and three untreated patients had stable HIV loads. Two patients spontaneously cleared the first infection, whereas HCV eradication was achieved in the others by pegylated-IFN-α (peg-IFN)-based treatment. At the second infection six patients were infected with a different genotype and showed a HCV genotype shift from one to four or vice versa, and nine patients were infected with the same genotype (all one).

In this cohort of 15 patients, plasma IL-12p40, IP-10, MIG and MIP-1β levels were increased upon primary acute HCV infection as compared to the pre-infection sample (Figs 2 and S1). As limited changes in HIV load during the subsequent sampling points were observed, these changes are not explained by HIV viral-load kinetics. No clear difference was observed between the cytokine patterns of patients with a high HIV viral load compared to those with a very low viral load. After HCV eradication these biomarkers normalized to the levels observed before primary acute HCV infection, although the decrease did not reach significance (Fig. 1). sIL-2R and IL-18 plasma levels were not significantly altered as a consequence of primary acute HCV infection, and neither IFN-γ nor IL-6 were detectable in the large majority of patients (Table S2). Two patients spontaneously cleared the virus, but their plasma cytokine profiles were similar to those of the 13 patients receiving peg-IFN-based therapy shortly after diagnosis.

Lower pro-inflammatory cytokine/chemokine levels upon subsequent HCV reinfection
Next, we assessed the inflammatory response during consecutive acute HCV reinfections. In contrast to primary acute HCV infection, plasmaconcentrations of the pro-inflammatory cytokines/chemokines IL-12p40, MIP-1β, MIG and IP-10 were not increased during the second acute HCV infection when compared to the HIV mono-infected stages before or after the first acute HCV infection (Fig. 2). These lower cytokine levels observed during the secondary acute HCV infections were not observed for sIL-2R. The plasma sIL-2R concentrations were increased after the resolution of the primary HCV infection. The sIL-2R levels increased weakly again during HCV reinfection (Fig. S1), albeit not significantly. Furthermore, the HCV RNA levels were significantly lower during the second acute HCV infection compared to the primary infection, with a median HCV RNA load of 69 000 IU ml⁻¹ (IQR 527–685 000) versus 707 000 IU ml⁻¹ (IQR 32 900–2 200 000, P=0.017). In contrast, the ALT levels were not significantly different, although they showed a declining trend when the second infection was compared to the first (median ALT 62 IU/L (IQR 40–264) versus 139 IU/L (IQR 34–992, P=0.152).

DISCUSSION

Despite the high cure rates of direct-acting antivirals in the treatment of HCV, patients in high-risk groups, such as injecting drug users and HIV-infected MSM, continue to transmit the virus and show frequent reinfections. Little is known about the immune response against HCV during reinfection after therapy-induced or spontaneous clearance of a first acute HCV infection. We assessed the inflammatory responses before, during and after primary HCV infection and during a subsequent acute HCV reinfection in HIV-positive MSM. Concentrations of pro-inflammatory cytokines IL-12p40, MIP-1β, MIG and IP-10 in plasma were increased during primary acute HCV infection as compared to the pre-infection state. However, we now show for the first time that these responses are not observed during the second acute HCV infection in the majority of patients.

We hypothesize that the weak pro-inflammatory cytokine responses observed during acute HCV reinfection may, on the one hand, compromise the chance of spontaneous clearance and, on the other, avoid damage to the infected liver, since strong memory responses may pose the risk of excessive immune-mediated toxicity. In this regard, MIP-1β, MIG, IP-10 and IL-12p40 are of interest as they bridge innate to adaptive immunity and are important for the generation of immune responses against HCV infections. IL-12p40, the beta-subunit of IL-12 produced by various immune cells, including liver resident macrophages (Kupffer cells) and dendritic cells, is important for the differentiation into T-helper 1 cells and NK cells leading to the induction of IFN-γ. In addition, the chemokines MIP-1β, MIG and IP-10 recruit inflammatory cells, such as CD4+ and CD8+ T cells, NK cells, plasmacytoid dendritic cells and monocytes, to the liver and promote both HCV clearance and fibrosis progression [4]. In line with our hypothesis, others observed recently that during acute HCV infection, high ALT and HCV RNA levels were associated with increased IP-10 levels, and that high HCV RNA levels were associated with increased MIP-1β levels. The authors suggested that IP-10 and MIP-1β may have a role in HCV immuno-pathogenesis starting early in acute
HCV infection [11]. In contrast to our findings, one study reported elevated IL-18 levels even upon reinfection in a small cohort of patients [12]. Although we did not find this increase overall in our cohort, a subgroup of 4 out of 15 patients did show substantial IL-18 production during reinfection. Therefore, larger datasets of prospective cohorts may further clarify the factors influencing these differences, and explain patient heterogeneity. Furthermore, it remains to be determined how the altered cytokine patterns influence the antiviral state leading to both lower HCV RNA loads and potentially decreased liver damage, as reflected by ALT levels during reinfection.

Several mechanisms may be responsible for the weak responses observed during acute HCV reinfection. Negative regulation by IL-10, TGF-β and regulatory T cells may be important, as we previously demonstrated that these regulatory mechanisms remain active years after therapy-induced eradication of chronic HCV infections [13]. Similar processes may occur in repeated acute HCV infections. Furthermore, in chimpanzees, repeated exposure to subinfectious HCV concentrations led to the suppression of HCV-specific T-cell responses [14]. It is possible that a delicate balance of silenced protective immunity and mild immunopathology exists during subsequent acute HCV reinfection, which mirrors reports on other infections, such as leishmaniasis and toxoplasmosis [15].

In summary, we now report for the first time that proinflammatory responses are weaker during acute HCV reinfection as compared to primary infection. However, the impact of these immunological differences on spontaneous viral clearance or immunopathology could not be investigated in the present study as most patients were treated after

**Fig. 1.** Plasma cytokine levels in healthy controls [healthy (n=10)], chronic HIV mono-infected patients [HIV (n=15)] with their first acute HCV infection [acute HCV/HIV (n=15)] and chronic HCV/HIV co-infected patients [chronic HCV/HIV (n=10)]. A Mann–Whitney test was used to compare the separate groups with each other. A Wilcoxon signed rank test was used for paired data. Horizontal bars are median.
their first infection, with spontaneous clearance not being waited for. Therefore, future research should focus on the impact of diminished immune activation during acute HCV reinfection on clinical outcomes, such as viral clearance and liver fibrosis.

METHODS

Plasma samples from HIV-positive patients with two consecutive acute HCV infections, from the Erasmus Medical Center Rotterdam and University Medical Center Utrecht, were retrospectively selected. Samples were stored at −80 °C. Only samples of HIV-positive patients who had not opted out of the ATHENA cohort were included in the study [16].

All patients had a chronic HIV co-infection and were diagnosed with a primary HCV infection between 2008 and 2014. HCV infection was diagnosed by a positive HCV RNA test [COBAS AmpliPrep-COBAS Taq-Man HCVv2 test CAP/CTM, Roche Molecular Systems, Indianapolis, USA (lower limit of detection 15 IU ml−1)]. To confirm that the first infection was acute, a documented negative HCV RNA, HCV antibody or a normal ALT (<45 U ml−1) within 1 year preceding the first positive HCV RNA sample had to be available. Consecutive plasma samples of the patients at four time points were selected in which the patient was HIV+HCV−, HIV+ accompanied by acute HCV infection (<6 months after first positive HCV RNA), HIV+HCV− (HCV PCR negative at least 4 weeks after treatment) and HIV+ accompanied by a second acute HCV infection (reinfection). HCV reinfection was confirmed by either genotype change, phylogenetic analysis or detectable HCV RNA after confirmed sustained virological response 24 weeks after the end of therapy or spontaneous clearance [HCV RNA negative and normal ALT (<45 U ml−1)]. Additionally, control plasma samples were used from healthy volunteers and from patients who were chronically infected for both HIV and HCV.

Plasma cytokine and chemokine levels were determined using Procarta Plex human cytokine/chemokine multiplex immunoassays (eBioscience, Vienna, Austria). Concentrations of eight distinct analytes were measured using the microsphere-based multiplex LUMINEX-1000 (Luminex Corporation, Austin, USA): interferon-γ (IFN-γ), interleukin-6 (IL-6), soluble IL-2 receptor (sIL-2R), IL-12, IL-18, C-X-C motif chemokine ligand-9 [CXCL9 or monokine induced by gamma IFN-(MIG)], CXCL10 [or IFN-γ-induced protein-10 (IP-10)] and C-C motif chemokine ligand-4 [CCL4 or macrophage inflammatory protein-1β (MIP-1β)]. Values are reported in pg ml−1.

Statistical analysis

Paired and non-paired samples were analysed using non-parametric tests. Graphs were constructed using GraphPad Prism 5. SPSS Statistics version 21 was used for statistical analysis.

Funding information

This work was supported by the Virgo consortium, funded by the Dutch government (project number FES0908).

Acknowledgements


Conflicts of interest


References


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