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Anti-inflammatory cytokines, pro-fibrogenic chemokines and persistence of acute HCV infection

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SUMMARY

Chemokines and cytokines play a vital role in directing and regulating immune responses to viral infections. Persistent hepatitis C virus (HCV) infection is characterized by the loss of anti-HCV cellular immune responses, while control of HCV infection is associated with maintenance of anti-HCV cellular immune responses. To determine whether plasma concentrations of 19 chemokines and cytokines controlling T-cell trafficking and function differed based on infection outcome, we compared them in at-risk subjects followed prospectively for HCV infection. Levels were compared over time in subjects who controlled HCV infection (Clearance) and subjects who developed persistent HCV infection (Persistence) at two time points during acute infection: (i) first viraemic sample (initial viraemia) and (ii) last viraemic sample in Clearance subjects and time-matched samples in Persistence subjects. At initial viraemia, increased pro-inflammatory tumour necrosis factor (TNF) plasma concentrations were observed in the Clearance group, while the plasma levels of anti-inflammatory interleukin (IL)-2, IL-10 and IL-13 were higher in the Persistence group. IL-13 was positively correlated with IL-2 and IL-10 at initial viraemia in the Persistence group. At the time of last viraemia, plasma levels of eotaxin, macrophage chemoattractant protein-4 (MCP-4), IL-5 and IL-10 were higher in the Persistence group and IL-10 and IL-5 levels were positively correlated. Collectively, these results suggest that the development of persistent infection is associated with an anti-inflammatory and pro-fibrogenic chemokine and cytokine profile that is evident at the onset of infection and maintained throughout acute infection.

Keywords

acute infection; chemokine; cytokine; hepatitis c virus

INTRODUCTION

Infection with hepatitis C virus (HCV) is a global public health concern with an estimated 170 million people infected worldwide and a prevalence rate of 1–2% in most countries [1]. Between 70% and 80% of infections are not spontaneously controlled, but instead develop into persistent infection [2]. Persistent HCV infection is a major aetiological factor in the development of chronic liver disease, the most common cause of hepatocellular carcinoma,

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CONFLICT OF INTEREST

There are no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

Study design – W.O.O, M.A.C, D.L.T and A.L.C; data acquisition – J.S.L; analysis and interpretation of data – W.O.O, M.A.C and A.L.C; drafting of manuscript – W.O.O; critical revision of manuscript – D.L.T and A.L.C.

and the most frequent indication for liver transplantation in the United States and many other countries [3]. Current treatment regimens result in control of viraemia in 45–80% of individuals, although these regimens are associated with significant side effects [4]. Therefore, identification of mechanisms that lead to spontaneous control of HCV infection before the development of persistent infection might facilitate development of novel therapies that decrease morbidity and mortality.

Chemokines are proteins released by a variety of cells during inflammation that act as chemotactic molecules for immune cells, while cytokines are immunomodulatory proteins that modulate effector functions of immune cells. These proteins direct and amplify immune responses, and some chemokine and cytokine levels differ in chronically infected individuals compared with healthy controls. For example, increased levels of CXCL10 (IP-10) [5], monocyte chemotactic protein-1 (MCP-1) [5,6], IL-10 [6] and eotaxin [7] have been detected in persistently infected individuals compared with healthy controls. Additionally, among persons with persistent infection, low serum levels of IP-10 prior to the initiation of pegylated interferon/ribavirin treatment have been demonstrated to be an independent predictor of sustained virological response following treatment [8]. However, these studies may highlight the consequences of chronic infection rather than chemokine and cytokine profile differences that promote chronic infection because subjects were assessed long after the outcome of infection was determined. A comparison of chemokine and cytokine levels during acute infection between persons who develop persistent infection and those who ultimately control viraemia is optimal for determining this. However, data on the chemokine and cytokine milieu during acute HCV infection in humans are limited, primarily due to the difficulty in identifying acutely infected subjects because most HCV infections are asymptomatic during the acute phase of infection.

In this study, we examined chemokine and cytokine profiles during acute infection at the time of initial viraemia and just prior to viral clearance in subjects who spontaneously control infection and in subjects who developed persistent infection. We chose initial viraemia to assess whether the initial response to HCV infection differs and the time of final viraemia to assess the profile closest to the time of infection control, enhancing the chances of finding a signature characteristic of control. We found increased levels of anti-inflammatory cytokines at initial viraemia in subjects who developed persistent infection, while increased levels of the pro-inflammatory cytokine TNF- α were observed in subjects who controlled infection. Similarly, this anti-inflammatory profile in subjects who progress to persistent infection was maintained late in acute infection as increased levels of anti-inflammatory and allergy-related immune signalling proteins were detected in persistently infected subjects compared with HCV controllers immediately prior to viral clearance. Taken together, these results demonstrate that the development of persistent HCV infection is associated with a predominantly anti-inflammatory chemokine and cytokine profile that is maintained throughout acute infection.

METHODS

Subjects

The Baltimore Before and After Acute Study of Hepatitis (BBAASH) cohort is a prospective study of injection drug users (IDU) at-risk for hepatitis C infection. Eligible participants have ongoing intravenous drug use and are seronegative for anti-HCV antibodies at enrolment. Written consent was obtained from each participant. Once enrolled, participants receive counselling to reduce intravenous drug use and its complications. Blood is drawn for isolation of serum, plasma and peripheral blood mononuclear cells (PBMC) in a protocol designed for monthly follow-up as previously described [9]. The study was approved by the Institutional Review Board at the Johns Hopkins School of Medicine. HCV

RNA measurements are used to identify the time of infection and to determine the outcome of infection. All participants with acute HCV infection are referred for evaluation for treatment. Subject demographic data are shown in Table 1.

Determination of infection outcome and subject selection

Control of HCV infection was defined as the absence of detectable viraemia for a period of >60 days. Persistent HCV infection was defined as continuous viraemia or recurrent viraemia with the same virus as confirmed by Core-E1 phylogenetic analysis, as described later. The midpoint between last positive and first HCV RNA-negative sample collection dates was used to determine infection duration in those who controlled. Fifty-three participants from the BBAASH cohort were selected for analysis based on the following inclusion criteria: 1) the initial HCV-positive sample was collected <365 days since the previous HCV RNA-negative sample, 2) gaps in follow-up following initial detection of HCV viraemia were <180 days, 3) HBsAg was undetectable and 4) subject was HIV negative.

HCV RNA Assays

Determination of HCV viral load—Total RNA was extracted from serum using a Qiagen MinElute Virus column (Qiagen, Valencia, CA) per manufacturer's instructions. To determine the concentration of HCV RNA in plasma, we used a quantitative RT-PCR assay (TaqMan[®] HCV analyte-specific reagent, Roche Molecular Diagnostics, USA). The generation of DNA amplification products was monitored on a Cobas TaqMan[®] Analyzer (Roche Molecular Diagnostics, USA). This assay has a lower limit of detection (LLOD) of 50 IU/mL. Samples with HCV RNA concentrations below the LLOD were assigned a value of 25 IU/mL.

Core-E1 phylogenetic analysis—Phylogenetic analysis of Core-E1 regions from RT-PCR products amplified from RNA extracted from plasma samples was performed as previously described [10].

Alanine aminotransferase (ALT) assay

Plasma ALT levels were determined by the Johns Hopkins Hospital clinical laboratory.

Measurement of chemokine and cytokine levels in human plasma samples

The MesoScale Discovery (Meso Scale Discovery, Gaithersburg MD) electrochemiluminescence multiplex assay was used to measure a total of 19 analytes in HCV RNA+ plasma specimens: eotaxin, interferon γ (IFN γ), interferon α (IFN α), interferon β (IFN β), IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IP-10, MCP-1, MCP-4, macrophage inflammatory protein (MIP-1), thymus and activation-regulated chemokine (TARC) and TNF- α . The assay was performed per the manufacturer instructions using 25 μ L of plasma from each time point. Plasma concentrations of analytes were determined using a standard curve obtained from reference standards for each analyte. Data were acquired using a SECTOR Imager SI2400 and results were analysed using the MesoScale Discovery Workbench software. Samples with values below the limit of detection of the assay were assigned a concentration of 0.5 pg/mL.

Assessment of anti-HCV immune responses

HCV pseudoparticle production and neutralization assays—HIV-HCV (HCV genotype 1a, H77; HCV genotype 1b, 1b35, GenBank accession no. (To be submitted upon acceptance)), and HIV-murine leukaemia virus (MLV) pseudoparticles (pp) containing the luciferase reporter gene were produced as described elsewhere [11,12]. Neutralization

assays were performed as previously described using heat-inactivated plasma or serum samples [13]. Samples at 12 months (± 1 month) of infection for persistently infected subjects or clearance subjects with initial infection durations >12 months were selected for analysis. The last sample with detectable viraemia was used for clearance subjects with initial infection durations <12 months. Pseudoparticle infection, indicated by luciferase activity, was measured in terms of relative light units (RLUs) in the presence of subject serum (RLUtest) vs average infection in the presence of two HCV-negative plasma or serum specimens (RLUcontrol). Percentage neutralization was calculated as $100 \times [1 - (\text{RLUtest} / \text{RLUcontrol})]$. The presence of nAb was noted when the 50% inhibitory dilution value was 1:50 for either the genotype 1a or 1b HCVpp. As a control, all subject serum neutralized MLVpp $<50\%$.

IFN γ ELISpot analysis—HCV-specific CD8 $^{+}$ T-cell responses were quantified by IFN-ELISpot assay as previously described [2]. Briefly, PBMC from subjects infected with genotype 1 viruses were screened for recognition of HCV-specific antigens using overlapping peptides and previously described optimal HCV epitopes. Pooled cytomegalovirus, Epstein–Barr virus, and influenza antigens and phytohemagglutinin were used as positive controls. A unique T-cell response was defined as recognition of a peptide by PBMC that was not recognized by PBMC collected at earlier time points.

Statistical analysis

Age, infection duration, HCV RNA levels and ALT activities between groups were compared using Student's t-test. Proportions were analysed using either a Fisher's exact test or Chi-squared test. A Wilcoxon rank-sum test was used for comparisons of log-transformed chemokine and cytokine concentrations between groups when $<50\%$ of the values were below the limit of detection. A Fisher's exact test was used to compare infection genotype as well as chemokine and cytokine concentrations when more than 50% of the values were below the limit of detection and samples were classified as either detectable or undetectable. Spearman rank correlation coefficients were determined to assess associations between chemokine and cytokine concentrations and CD8 $^{+}$ T-cell responses. Differences were considered statistically significant when $P < 0.05$ for all analyses except Spearman rank correlations for which Holm's Sequential Bonferroni method was used to adjust for multiple comparisons.

RESULTS

Chemokine and cytokine levels at initial viraemia

The blood concentrations of a panel of 19 chemokines and cytokines were determined on initial HCV viraemia samples from 35 subjects who subsequently developed persistent HCV infection (Persistence) and 18 subjects who eventually controlled an HCV infection (Clearance). The first sample with detectable HCV RNA from all subjects in the Clearance and Persistence groups was used for comparison of chemokine and cytokine concentrations at the time of initial viraemia. No differences were detected in the Persistence and Clearance groups in the age at seroconversion, gender, race, HCV infection genotype, HCV RNA levels, plasma ALT activity, duration of infection at time of analysis, proportion of subjects with detectable neutralizing antibody responses and breadth of CD8 $^{+}$ T-cell responses (Table 1). The IL28B genotype for each subject is listed in Supplementary Table S1. Blood chemokine (eotaxin, IL-8, IP-10, MCP-1, MCP-4, MIP-1 and TARC) concentrations in initial viraemia samples were similar in the Persistence and Clearance groups (Table 2), suggesting that the levels of these immune trafficking proteins early in acute infection are not predictive of infection outcome. Additionally, the blood concentrations of a number of cytokines, including IL-1, IL-2, IL-4, IL-5, IFN γ , IFN α and IFN β , were similar in the

Persistence and Clearance groups, indicating that the levels of these immune signalling proteins at initial viraemia are not associated with infection outcome. Higher plasma levels of TNF- α were observed at initial viraemia in subjects who clear ($P=0.003$; Table 2) compared with subjects who progress to persistent infection. Conversely, increased plasma concentrations of IL-2 ($P=0.048$; Table 2), IL-10 ($P=0.046$) and IL-13 ($P=0.017$) were detected at initial viraemia in subjects who progress to persistent infection compared with subjects who control infection. The levels of these cytokines and chemokines were not significantly different between subjects with and without detectable neutralizing antibody responses during the first 12 months of infection and were not associated with the breadth of CD8+ T-cell responses (Table 3), suggesting that the effect is not due to these downstream adaptive immune responses. These results suggest that spontaneous control of HCV infection is associated with increased blood concentrations of TNF- α at initial viraemia and lower levels of IL-2, IL-10 and IL-13 at initial viraemia.

In a previous study, we detected higher HCV RNA levels at initial viraemia in subjects who control infection compared with subjects who progress to persistent infection [14]. Therefore, Spearman rank-order correlations between the levels of chemokines and cytokines at initial viraemia and HCV RNA concentrations were assessed for each outcome group to investigate whether the release of these chemokines and cytokines is differentially associated with HCV RNA levels based on infection outcome at initial viraemia. No chemokine or cytokine was significantly correlated with IL28B genotype or HCV RNA levels at initial viraemia, suggesting that IL28B status and the magnitude of viraemia do not influence the production or release of these chemokines and cytokines (Supplementary Table S2 and S3).

Correlations between divergent chemokine and cytokine concentrations at initial viraemia

Correlations between the concentrations of cytokines at initial viraemia that differed based on infection outcome were determined to assess the relationship between the secretion of these chemokines and cytokines early in infection in both Persistence and Clearance groups. No statistically significant correlations between TNF- α , IL-2, IL-10 and IL-13 were observed in the Clearance group (Table 4). However, IL-13 was significantly positively correlated with IL-2 and IL-10 in the Persistence group, suggesting coordinated secretion of these cytokines at initial viraemia in subjects who progress to persistent infection.

Chemokine and cytokine levels at last viraemic sample

We hypothesized that chemokine and cytokine profiles might be unique in those who control HCV infection as the infection is being controlled. Therefore, chemokine and cytokine measurements were also performed on the last HCV RNA-positive sample in 18 subjects who control infection as well as a time-matched (± 60 days) sample from 18 subjects who develop persistent infection. The age at seroconversion, gender, race, HCV infection genotype, HCV RNA levels, plasma ALT activity and duration of infection at time of analysis were similar in the Persistence and Clearance groups (Table 1). Of the chemokines, blood concentrations of eotaxin ($P=0.042$, Table 2) and MCP-4 ($P=0.017$) were higher in time-matched samples from persistently infected subjects compared with the last viraemic sample obtained from subjects who control infection. No differences in the levels of IL-8, IP-10, MCP-1, MIP-1 and TARC were observed in the Persistence and Clearance groups. These data suggest that successful control of HCV infection is associated with lower eotaxin and MCP-4 levels late in acute infection.

Likewise, no differences in the levels of most cytokines was observed, including IFN- α , IFN- γ , IFN- λ , IL-1, IL-4, IL-6, IL-12p70, IL-13 and TNF between the Persistence and Clearance groups. However, the levels of IL-2 ($P=0.049$), IL-5 ($P=0.042$) and IL-10 ($P=$

0.018) were higher in time-matched samples from persistently infected subjects compared with final viraemia samples obtained from subjects who control infection. These results indicate that lower levels of IL-2, IL-5 and IL-10 late in acute infection are associated with control of HCV infection.

The levels of chemokines and cytokines were compared between outcome groups and correlations between the levels of chemokines and cytokines at the time of last viraemia and HCV RNA concentrations were assessed for each outcome group in order to investigate whether the release of these chemokines and cytokines is associated with HCV RNA levels or IL28B genotype based on infection outcome. No significant correlation was observed between the concentration at final viraemia of any chemokine or cytokine tested and HCV RNA or IL28B genotype in either the Clearance or the Persistence group (Supplementary Table S1 and S2).

Correlations between divergent chemokine and cytokine concentrations at final viraemia

Spearman correlations between the concentrations of cytokines that differed at final viraemia based on outcome were determined to assess whether the secretion of these chemokines and cytokines at final viraemia was coordinated. No significant correlations were observed between MCP-4, IL-2, IL-5 and IL-10 in either the Clearance or the Persistence group (Table 5).

DISCUSSION

We performed detailed studies of individuals followed prospectively who acquired HCV infection to assess the innate immune response regardless of whether they developed symptoms and prior to the development of an adaptive immune response. We demonstrated that successful immunological control of HCV is associated with higher concentrations of the pro-inflammatory cytokine TNF and lower levels of anti-inflammatory cytokines (IL-5, IL-10, IL-13, MCP-4 and eotaxin) (Fig. 1). These differences in the innate immune response are evident at the first measured viraemia and at the time of viral control in those who control infection. The differences were not due to IL28B genotype and may be due to other inherent host differences or factors related to the infecting virus.

Our results demonstrate that the level of the pro-inflammatory cytokine TNF at the onset of acute HCV infection is higher in subjects who eventually control infection compared with subjects who develop persistent infection. *In vitro* stimulation of Kupffer cells by HCV proteins has been shown to result in production of TNF [15]. Therefore, it is possible that Kupffer cells in individuals who control HCV infection are able to more efficiently respond to HCV viral proteins at the onset of infection, increasing the likelihood of infection control.

Similarly, an increased number of individuals in the Persistence group had detectable levels of IL-2 at initial and final viraemia compared with the Clearance group. In addition to the T-cell proliferative activity of IL-2, IL-2 also results in the differentiation and proliferation of CD4⁺CD25⁺ natural regulatory T cells (Treg) [16] and the predominant phenotype of IL-2 knockout mice is autoimmunity [17]. A number of studies in humans and chimpanzees have demonstrated increased levels of Tregs in persistently infected subjects compared with recovered subjects or healthy controls [18–21]. Tregs from HCV-infected subjects have been shown to decrease anti-HCV cellular immune responses *in vitro* [22]. Therefore, the increased IL-2 levels we observed during acute infection when the virus ultimately persists may contribute to an increase in Treg populations in persistently infected individuals and the decreased HCV-specific cellular immune responses observed during persistent infection.

We also demonstrated increased levels of IL-10 and IL-13 at initial viraemia and IL-5 at final viraemia in subjects who develop persistent infection. These three cytokines mediate an anti-inflammatory, Th2-type immune response characterized by humoral responses and inhibition of macrophage function. Given that the concentration of none of these cytokines was higher in subjects with neutralizing antibody responses detected during the first 12 months of infection, the main effect of these cytokines may be inhibition of immune function. IL-10 is an anti-inflammatory cytokine that has been shown to directly downregulate MHC class II and costimulatory molecules on antigen-presenting cells [23] and to inhibit T-cell activation via suppression of the costimulatory molecules CD28 and Inducible Costimulator of T-cell Signalling (ICOS) [24]. Indeed, treatment of persistently infected individuals with IL-10 resulted in a decrease in the levels of anti-HCV IFN - producing CD8+ T-cell responses and an increase in HCV RNA levels [25]. In our study, the breadth of T-cell responses in the Persistence group (median 4 responses) was similar to the Clearance group (median 5 responses) and IL-10 was not significantly correlated with T-cell response breadth even though the levels of IL-10 during acute infection were higher in individuals who develop persistent infection at initial and final viraemia. However, maintenance of increased IL-10 levels during acute infection in subjects who develop persistent infection may promote persistence of HCV infection through IL-10-mediated suppression of T-cell activity and contribute to the eventual decline of T-cell responses observed in these subjects [2].

IL-5 has been reported to promote the differentiation and activation of eosinophils [26], while IL-13 has been implicated in activation of mast cells and eosinophils during allergic reactions. Increased IL-13 has also been observed during fibrosis development following schistosoma infection [27]. While IL-5 and IL-13 have been implicated in the pathogenesis of allergic diseases, the mechanism by which these cytokines could contribute to persistent HCV infection remains to be elucidated. Interestingly, the levels of these cytokines were strongly correlated with one another at initial viraemia, suggesting that coordinated regulation of these cytokines at the onset of infection may lead to an ineffective Th2 immune response, thereby resulting in the development of persistent infection.

In addition to the cytokines IL-5 and IL-13, we identified two allergy-associated chemokines that were increased late in acute infection in persistently infected subjects compared with subjects who control HCV infection; eotaxin and MCP-4. Eotaxin has been demonstrated to play a role in eosinophil trafficking to sites of allergic inflammation [28], while MCP-4 has been shown to contribute to the pathogenesis of rheumatoid arthritis [29,30]. While the exact mechanistic role of MCP-4 and eotaxin in acute HCV infection remains unclear, a previous study demonstrated an association between increasing levels of eotaxin and progression of liver fibrosis [31]. Therefore, the increased eotaxin levels observed in persistently infected subjects late in acute infection might be a biomarker for the transition from acute to chronic infection and the beginning of the progression of liver disease in these individuals. Taken together, increased levels of allergy-related chemokines and cytokines might be biomarkers for the development of liver disease.

Previous research demonstrated that low levels of the chemokine IP-10 are associated with successful interferon and ribavirin treatment of HCV infection [8]. Therefore, we included IP-10 in our analysis to determine whether lower IP-10 levels at the onset of infection or late in acute infection were associated with spontaneous control of infection. No difference in IP-10 level in the two outcome groups was observed, suggesting that unlike in treatment associated control of infection, IP-10 levels during acute infection are not predictive of spontaneous control.

One limitation of this study is that our monthly sampling interval may not have been sufficiently frequent to detect peak levels of cytokines or chemokines. As acute HCV infection is predominantly asymptomatic, frequent monitoring of at-risk individuals is the only way to identify acute HCV infection as it presents most commonly. However, more frequent assessment of HCV infection status in at-risk individuals is not feasible and no small animal model of HCV infection exists so determining cytokine and chemokine levels during the first few days of infection for every subject is not uniformly possible. Additionally, the findings of this study may be limited by the difficulty in measuring cytokine levels systemically when cytokines act in a paracrine fashion with increased levels localized to the site of release. More informative assessments of cytokine levels during acute HCV infection may depend upon technological advances or assessment of cytokine levels in liver tissue biopsy samples. However, we used a highly sensitive assay for analysing cytokine levels in human blood and did detect some differences in cytokine levels based on outcome. In addition, we previously determined that the cytokine IL-18 is produced at high levels in acute infection regardless of infection outcome but at different levels following control of infection [32], thereby demonstrating the feasibility of cytokine detection in plasma.

Collectively, the results of our study demonstrate that clearance of HCV infection is associated with increased TNF early in acute infection. Conversely, persistence of HCV infection is associated with increased secretion of anti-inflammatory cytokines both early and late in acute infection as well as increased levels of fibrosis associated-chemokines. Detailed study of the regulatory mechanisms controlling production and secretion of these chemokines and cytokines during persistent infection may identify potential therapeutic targets to prevent persistent HCV infection or liver fibrosis in those who can not successfully be cured of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HCV	hepatitis c virus
IDU	intravenous drug user
IL	interleukin
IP-10	CXCL10
LLOD	lower limit of detection
MCP-1	monocyte chemotactic protein-1
MCP-4	macrophage chemoattractant protein-4
PBMC	peripheral blood mononuclear cells
TARC	thymus and activation-regulated chemokine
TNF	tumour necrosis factor

Treg regulatory T cell

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Fig. 1.

Chemokines and cytokines with divergent concentrations at initial and final viraemia in Clearance and Persistence subjects. IDUs were longitudinally assessed for HCV infection status and the first sample with detectable viraemia was selected for analysis in both groups. For final viraemia, the last sample with detectable viraemia was used in the Clearance groups, while samples from subjects with persistent infection were time-matched (± 60 days) with final viraemia time points in the Clearance group.

Table 1
Demographic and infection data of persistence and clearance subjects included in initial and final viraemia analyses

	Initial Viraemia			Final Viraemia		
	Persistence	Clearance	P	Persistence	Clearance	P
<i>N</i>	35	18	–	18	18	–
Age [*]	25.5	25	0.62	27	25	0.45
Female (%)	38.9	61.1	0.32	34.3	61.1	0.18
Caucasian (%)	88.6	100	0.49	83.3	100	0.23
HCV genotype (%)						
1a	77.1	55.0	0.16	88.2	61.1	0.18
1b	2.9	15.0		5.6	16.7	
2b	8.6	5.0		5.6	5.6	
3a	11.4	25.0		0.0	16.7	
Infection duration [†]	71 (21–108)	47 (21–67)	0.31	129 (98–296)	75 (52–318)	0.26
HCV RNA [‡]	5.5 (4.5–6.7)	6.4 (5.3–6.8)	0.35	5.9 (4.9–6.6)	4.2 (3.3–6.5)	0.16
ALT [§]	85 (27–139)	43 (33–347)	0.82	119 (42–263)	37 (25–310)	0.19
Detectable nAb [¶] (%)	48.3	61.1	0.59	–	–	–
T-cell breadth	4 (3–9)	5 (3–9)	0.81	–	–	–
<i>IL28B</i> ^{**} (%)						
<i>CC</i>	40.0	55.6	0.20	38.9	55.6	0.10
<i>CT</i>	45.7	44.4		38.9	44.4	
<i>TT</i>	14.3	0.0		22.2	0.0	

^{*} yr at seroconversion, median (IQR);
[†] days, at time of analysis, median (IQR);
[‡] IU/mL, Log10, median (IQR);
[§] median (IQR), U/dl;
[¶] detectable antigenotype 1a or antigenotype 1b nAb;
^{||} number of CD8+ T-cell epitopes recognized in an ELISpot assay during the 1st year of infection;

IL28B SNP rs12979860 genotype.

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Table 2

Chemokine and cytokine concentrations* at initial and final viraemia in persistence and clearance groups

Analyte	Initial Viraemia			Final Viraemia		
	Persistence	Clearance	<i>P</i> [†]	Persistence	Clearance	<i>P</i> [†]
Eotaxin	926 (782–1373)	739 (594–1036)	0.090	1047 (759–1380)	589 (417–912)	0.042
IFN α	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.272	0.5 (0.5–0.5)	0.5 (0.5–0.5)	1.000
IFN γ	316 (89–710)	262 (92–994)	0.897	493 (93–1296)	157 (50–6473)	0.910
IFN δ	1.7 (0.5–2.4)	0.8 (0.5–1.9)	0.139	1.6 (0.5–3.6)	1.5 (0.5–4)	0.833
IL-1	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.657	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.706
IL-2	0.5 (0.5–1.3)	0.5 (0.5–0.5)	0.048	0.5 (0.5–2.1)	0.5 (0.5–0.5)	0.049
IL-4	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.358	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.572
IL-5	1.2 (0.5–2.4)	0.5 (0.5–1.7)	0.126	0.8 (0.5–1.9)	0.5 (0.5–1.7)	0.042
IL-6	1.4 (0.5–2.7)	1.5 (0.7–2.9)	0.075	1.5 (0.5–2)	1.6 (0.5–3.9)	0.352
IL-8	4.1 (2.9–6.0)	5.7 (3.5–8.9)	0.266	4.9 (3.3–6.2)	5.4 (3.6–8.9)	0.448
IL-10	4.7 (2.9–8.3)	3.3 (0.9–5.9)	0.046	5.8 (4.2–9)	3.8 (0.5–5.5)	0.018
IL-12 p70	0.5 (0.5–0.7)	0.5 (0.5–0.5)	0.080	0.8 (0.5–25.2)	0.5 (0.5–0.5)	0.099
IL-13	0.5 (0.5–14.4)	0.5 (0.5–0.5)	0.017	3.1 (0.5–13.1)	0.5 (0.5–0.5)	0.080
IP-10	836 (315–1339)	1101 (403–3208)	0.211	1288 (479–1950)	1096 (457–1380)	0.438
MCP-1	350 (292–452)	381 (308–458)	0.368	324 (257–417)	339 (295–457)	0.779
MCP-4	389 (270–551)	324 (244–521)	0.453	437 (339–550)	257 (240–398)	0.017
MIP-1	115 (87–209)	112 (80–216)	0.978	135 (107–204)	93 (79–117)	0.151
TARC	256 (159–514)	305 (137–670)	0.718	269 (162–832)	166 (135–316)	0.077
TNF- α	5.3 (3.3–7.6)	8.7 (6–11.8)	0.003	6.1 (4.6–8.5)	6.9 (5.5–11)	0.261

* median, (IQR); pg/mL; LL OD = 0.5 pg/mL;

[†] Wilcoxon rank-sum test or Fisher's exact test.

Table 3

Association of chemokine and cytokine concentrations* at initial viraemia with neutralizing antibody responses and breadth of CD8+ T-cell responses

Analyte	nAb detected during 1st 12 months [†]		CD8+ T-cell breadth [§]	
	No	Yes	P [‡]	P [¶]
Eotaxin	858 (657–1030)	832 (638–1137)	0.823	–0.092
IFN α	0.5 (0.5–0.5)	0.5 (0.5–0.5)	1.000	0.000
IFN	521 (93–1904)	179 (42–725)	0.533	0.209
IFN	1.5 (0.5–2)	1.4 (0.5–1.8)	1.000	–0.335
IL-1	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.350	0.069
IL-2	0.5 (0.5–0.5)	0.5 (0.5–2.2)	0.487	–0.219
IL-4	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.350	–0.212
IL-5	0.5 (0.5–1.8)	0.5 (0.5–2.4)	1.000	–0.141
IL-6	1.5 (0.5–2.8)	0.5 (0.5–2.6)	0.651	–0.230
IL-8	4.6 (3.0–8.3)	5.4 (3.0–6.4)	0.881	–0.047
IL-10	4 (1.8–7.7)	4.7 (2.7–8.8)	0.463	–0.048
IL-12 p70	0.5 (0.5–1)	0.5 (0.5–6.8)	1.000	0.155
IL-13	0.5 (0.5–2.2)	0.5 (0.5–15.3)	0.543	0.103
IP-10	1161 (433–1943)	747 (291–1298)	0.205	0.012
MCP-1	357 (300–458)	337 (301–397)	0.437	–0.043
MCP-4	412 (273–521)	300 (233–476)	0.086	–0.263
MIP-1	119 (82–272)	108 (80–202)	0.400	0.107
TARC	342 (187–493)	194 (133–670)	0.190	0.070
TNF	6.5 (4.7–8.6)	5.3 (3.2–8.4)	0.397	0.144

* median, (IQR); pg/mL; LOD = 0.5 pg/mL;

[†] detectable antigenotype 1a or antigenotype 1b nAb;

[‡] Wilcoxon rank-sum test or Fisher's exact test;

[§] number of CD8+ T-cell epitopes recognized in an ELISpot assay during the 1st year of infection;

[¶] Spearman correlation between cytokine and chemokine concentrations at initial viraemia and CD8+ T-cell breadth.

Table 4

Correlations between divergent cytokine concentrations at initial viraemia based on outcome

	Outcome	IL-2		IL-10		IL-13	
		*	P	*	P	*	P
TNF	Persistence	-0.228	0.187	-0.113	0.515	-0.200	0.247
	Clearance	0.378	0.099	0.442	0.055	-0.111	0.635
IL-2	Persistence			0.324	0.057	0.765	2E-7 [†]
	Clearance			0.341	0.138	-0.096	0.681
IL-10	Persistence					0.437	0.009 [†]
	Clearance					0.197	0.399

* Spearman correlation coefficient;

[†] statistically significant by Holm Sequential Bonferroni method.

Table 5

Correlations between divergent chemokine and cytokine concentrations at final viraemia based on outcome

Outcome	MCP-4		IL-2		IL-5		IL-10	
	*	P	*	P	*	P	*	P
Eotaxin Persistence	0.461	0.061	0.306	0.226	0.324	0.200	-0.014	0.951
Clearance	0.222	0.369	0.172	0.487	0.383	0.114	0.342	0.161
MCP-4 Persistence			-0.102	0.687	0.430	0.082	-0.150	0.559
Clearance			0.349	0.151	0.224	0.365	0.067	0.786
IL-2 Persistence					-0.167	0.503	0.402	0.096
Clearance					0.111	0.656	0.454	0.056
IL-5 Persistence							0.169	0.497
Clearance							0.574	0.013

* Spearman correlation coefficient.