Detection of host immune responses in acute phase sera of spontaneous resolution versus persistent hepatitis C virus infection

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Prior to the identification of hepatitis C virus (HCV), transfusion-transmission was common. Viral transmission in subjects with a known date of infection allows the study of the immune responses to acute HCV infection. We analysed 39 soluble immune factors in serum samples from subjects with transfusion-transmitted HCV. Dynamic expression kinetics of interferon gamma-induced protein 10 (IP-10), tumour necrosis factor-alpha and interleukin (IL)-10 were observed during acute HCV infection. Serum IP-10 was the only analyte that was significantly elevated in HCV resolvers compared with uninfected controls. In individuals who progressed to chronic HCV elevated levels of IP-10 and IL-10 coincided with first significant alanine aminotransferase elevation and remained elevated during the first year of acute HCV infection. In addition to monitoring lack of reduction in viral load, serum levels of IP-10 and IL-10 expression during acute HCV infection may be useful biomarkers to predict the progress to chronic HCV.

In the past 20 years since its identification, hepatitis C virus (HCV) has emerged as a major aetiological agent of liver disease throughout the world (Armstrong et al., 2006; Lavanchy, 2009). Of the persons acutely infected with HCV, 20–30 % spontaneously recover with clearance of viraemia shortly following seroconversion, while 70–80 % develop persistent infection (Alter, 2007; Marcellin, 1999; Mosley et al., 2008). However, for a given infected individual there is currently no way of predicting prognosis, including early clearance or progression to chronic HCV infection. Investigations into acute HCV infection are critical to elucidating the pathogenetic mechanisms underlying spontaneous clearance versus persistence of infection, yet this area of research has been severely hindered by the difficulty of identifying acutely infected persons as most are asymptomatic during this period (Alter, 1997; Busch, 2001; Selvarajah et al., 2010). Soluble immune factors or other biomarkers associated with acute HCV pathogenesis are not well defined, and very little is known about the early events in virus–host interactions that determine spontaneous HCV resolution versus progression to chronic HCV infection. Most studies have examined soluble immune factors and biomarkers in chronically infected individuals to evaluate correlations with treatment-induced resolution of HCV (Butera et al., 2005; Cacciarelli et al., 1996; Casrouge et al., 2011; Yoneda et al., 2011a, b). Researchers have also looked at biomarkers in HCV-infected individuals with previously resolved or persistent infections, or those who progressed to liver cirrhosis or hepatocellular carcinoma (Berres et al., 2011; Zeremski et al., 2009). These studies have generally been conducted on samples collected long after acute HCV infection has occurred, and hence the findings do not provide information on the early events following acute HCV infection that clearly play a critical role in establishing the parameters of persistent HCV infection and the resulting complications. However, in a recent study Zeremski et al. (2011) examined CXCR3- and CCR5-associated chemokines during acute HCV infection, but no differences in these chemokines were detected between HCV resolvers and those who would progress to chronic infection.

The archived samples from the Transfusion-Transmitted Viruses Study (TTVS) provide a unique opportunity to study acute HCV infection following transfusion-transmission with a known date of HCV infection, especially facilitated by the large number of serial samples from infected individuals. The main aims of the study were twofold: first to investigate the differential changes in cytokine, chemokine...
and growth factors that occur following acute HCV infection compared to transfused-uninfected controls; second, to identify biomarkers of infection outcome in the serum of individuals who spontaneously resolve HCV infection and in individuals who progress to chronic HCV infection.

Fourteen individuals acutely infected with either HCV genotype 1a or 1b virus were selected. Of these individuals, six spontaneously resolved their infection (resolvers) and eight progressed to chronic HCV infection (non-resolvers) (Table 1) (Mosley et al., 2008; Seeff et al., 2001). Our previous studies showed that clinical symptoms had a strong correlation to the outcome of disease. Among the six resolvers, three were symptomatic, two were icteric and only one individual was asymptomatic. In contrast, among the eight HCV chronic only one individual was symptomatic and seven were asymptomatic. There were 7–23 blood samples collected per patient (spanning up to 295 days) among the six HCV-infected individuals who resolved infection and 9–36 samples (spanning up to 365 days) from each of the eight HCV-infected individuals who progressed to chronic infection. As controls for the cytokine, chemokine and growth factor analysis we tested serial samples from 12 individuals collected for 6 months following transfusion with HCV-negative blood (uninfected controls) (three to eight samples per patient; spanning up to 150 days).

There was no significant difference in the peak viral load among HCV resolvers (6.34 log10 IU ml−1) compared with non-resolvers (4.83 log10 IU ml−1). However, at 16 weeks post-infection (p.i.) the viral load among HCV resolvers showed a three log reduction (from 6.34 to 3.0 log10 IU ml−1; P=0.002) in contrast to no significant change in viral load among non-resolvers (4.83 to 4.63 log10 IU ml−1; P=0.33) (Fig. 1). All HCV resolvers had undetectable levels of HCV RNA in the serum within 6–9 months p.i., while in individuals who progressed to chronic HCV infection the viral load was significantly higher throughout the first year of follow-up (Fig. 1; Table 1). A recent study also showed a rapid decline in viral load with 2.2 log10 IU ml−1 viral load drop within the first 100 days of infection, which was strongly associated with spontaneous clearance of acute HCV in human immunodeficiency virus (HIV) co-infected individuals (Thomson et al., 2011).

Multiplexed bead array assays were performed on serial post-transfusion TTTS specimens in order to investigate cytokine, chemokine and growth factor changes that occur following HCV infection. One hundred and thirty serial serum samples from HCV resolvers and 176 serial samples from HCV non-resolvers and 72 serial samples from 12 uninfected controls also from the TTTS cohort were assayed. Thirty-nine soluble cytokines, chemokines and growth factors in serum were examined in order to determine significant differences among the HCV resolvers and non-resolvers relative to uninfected controls. Serum samples were assayed using the high-sensitivity LincoPlex kit (Millipore) for interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p70, IL-13, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)-α, and the standard-sensitivity MilliPlex Map kit (Millipore) for epidermal growth factor (EGF), Eotaxin, fibroblast growth factor-2, Fractalkine, Flt-ligand, G-CSF, growth-related oncogene, IFN-α2, IL-1α, IL-1Rα, IL-3, IL-9, IL-12 (p40), IL-15, IL-17, interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein (MCP)-1, MCP-3, macrophage-derived chemokine, macrophage inflammatory protein (MIP)-1α, MIP-1β, soluble interleukin-2 receptor alpha, soluble CD40 ligand, transforming growth factor-alpha, TNF-β and vascular endothelial growth factor.

In our detailed analysis of cytokine, chemokine and growth factor dynamics we aligned the serial samples from the different subjects based on the first significant alanine aminotransferase (ALT) elevation (≥80 IU 1−1), and this time point was denoted as T0 (Fig. 1). The mean time from exposure (transfusion of HCV-infected unit) to first significant ALT elevation among the six HCV resolvers was 54 days, whereas it was 49 days in individuals who progressed to chronic HCV infection. There was no significant difference in first significant ALT levels in subjects who cleared HCV compared to HCV non-resolvers (Table 1). A mean basal ALT level of 16 IU 1−1 was detected over the course of 150 days post-transfusion in the uninfected controls, confirming that individuals in the control group were not transfused with HCV, hepatitis B virus (HBV) or any liver ALT elevating agents. The alignment of serial samples from subjects based on the first significant ALT elevation allowed us to separate the acute HCV infection period into two phases – a pre-ALT phase and a post-ALT phase. The pre-ALT phase included samples taken before the first significant ALT elevation and consisted of three to five samples per individual. The post-ALT phase was further divided into early post-ALT and late post-ALT phases. The early post-ALT phase included the first significant ALT sample as well as samples collected for up to 16 weeks following transfusion-transmission of HCV and consisted of 6–12 samples per individual. The 16-week cut-off was used to determine changes that occurred coincidently with viral load decline observed in spontaneous HCV resolvers. The late post-ALT phase in spontaneous HCV resolvers included samples collected from 17 up to 40 weeks following transfusion-transmission in spontaneous HCV resolvers or 50 weeks in non-resolvers. We first calculated the mean concentrations of cytokines, chemokines and growth factors from all serial serum samples from each subject within the subgroups of pre-ALT, early post-ALT or late post-ALT. We then quantified the median differences in expression of the individual analytes for all subjects within each of the groups. The cytokines, chemokines and growth factors were calculated and compared to controls using the Kruskal–Wallis test followed by Dunn’s post-test to correct for multiple comparisons (Fig. 2).
**Table 1.** Demographic and clinical details of HCV-infected individuals (acute phase)

<table>
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<tr>
<th>Subject ID#</th>
<th>Age</th>
<th>Gender</th>
<th>Genotype</th>
<th>First significant ALT elevation IU l⁻¹</th>
<th>First significant ALT elevation days since transfusion</th>
<th>HCV RNA at first significant ALT elevation in log IU ml⁻¹</th>
<th>HCV RNA level 6–9 months after HCV infection in log IU ml⁻¹</th>
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First significant ALT elevation, mean days since transfusion among HCV resolved individuals is 54 days and for chronic HCV is 49 days since transfusion. ND, Not detected.

*Subject ID# 1081; viral load at 6 months was 4.80 log₁₀ IU ml⁻¹ and at 9 months was 0.66 log₁₀ IU ml⁻¹; however, based on ALT levels and previously published data (Mosley et al., 2008) the individual progressed to chronic HCV infection.
Elevated expression of IP-10 was detected in the pre-ALT (497.3 pg ml\(^{-1}\)) and early post-ALT (925.9 pg ml\(^{-1}\)) phases in spontaneous HCV resolvers compared with uninfected controls (216.1 pg ml\(^{-1}\)) (Fig. 2). The IP-10 expression level in the late post-ALT phase in two of five HCV resolvers, denotes a downward trend toward baseline.
IP-10 level as the HCV RNA is cleared in these individuals. However, in HCV non-resolvers the IP-10 level was not significant in the pre-ALT phase (375.6 pg ml\(^{-1}\)), but increased to significant levels during the early post-ALT phase (823.9 pg ml\(^{-1}\)) and remained significantly high in the late post-ALT phase (732.9 pg ml\(^{-1}\)) compared with uninfected controls. IP-10, also known as CXCL10, is a potent chemoattractant for activated Th1 lymphocytes (adaptive immunity) and natural killer cells (innate immunity). Elevated IP-10 is also a prominent feature of the acute phase of several viral infections, including HIV, West Nile virus and influenza (de Jong et al., 2006; Stacey et al., 2009; Tobler et al., 2008). A recent study described an antagonist form of IP-10 as a biomarker of HCV treatment-response in chronically infected individuals and possible cause of treatment failure in these individuals (Casrouge et al., 2011).

We observed a modest increase of TNF-\(\alpha\), a pro-inflammatory cytokine, in the serum during the early post-ALT phase in HCV non-resolvers [10.5 vs 5.5 pg ml\(^{-1}\)] when compared with uninfected controls (Fig. 2). TNF-\(\alpha\) expression was transient but it is known to be a strong stimulant of downstream immune response events that could play a critical role even long after TNF-\(\alpha\) expression declines. However, TNF-\(\alpha\) detection in serum coincident with ALT may also indicate inflammatory response stimulated by liver damage. We did not observe significant

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**Fig. 2.** Detection of serum IP-10, TNF-\(\alpha\) and IL-10 in HCV-infected recipients compared to transfused-uninfected controls. The panel on the left-hand side shows changes in serum soluble factors of HCV resolvers, and the panel in the middle shows serum soluble factors in HCV non-resolvers. The panel on the right-hand side shows median trends of IP-10, TNF-\(\alpha\) and IL-10 levels shown in the graphs on the left-hand side and middle panel for HCV resolvers (red line) and HCV non-resolvers (blue line). Levels of IP-10, TNF-\(\alpha\) and IL-10 are shown on the y-axis as pg ml\(^{-1}\). The serum levels of the proteins were detected at three different phases (pre-ALT, early post-ALT and late post-ALT) and compared to uninfected controls. Kruskal–Wallis test followed by Dunn’s post-test was used for the statistical analysis. Significant \(P\)-values are highlighted using dotted lines and ‘*’ on top of each graph.
TNF-α levels in HCV resolvers compared to uninfected controls.

We detected significant increases in the levels of IL-10 during the early post-ALT phase in HCV non-resolvers compared with uninfected controls (24.0 vs 8.1 pg ml⁻¹) that remained elevated in the late post-ALT phase (29.2 vs 8.1 pg ml⁻¹) (Fig. 2). There was no significant increase in the levels of IL-10 in HCV resolvers compared to uninfected controls. Interestingly, among all the analytes the IL-10 expression level in the late post-ALT phase was significantly different between HCV resolvers and non-resolvers (5.1 vs 29.2 pg ml⁻¹, respectively).

Stacey et al. (2009) described a large study on cytokine and chemokine responses in serum during the first 40 days of acute HIV, HCV and HBV infection. They found that compared to HIV the cytokine response to HCV was less striking, which is similar to what we observed in the pre-ALT phase in HCV-infected individuals. The study detected elevations in TNF-α, IL-18, IP-10, MIP-1β, IL-10, IL-22, RANTES, IFN-γ and IL-6 in most but not all individuals (six to eight out of ten) and the serum response in HCV-infected individuals was not segregated based on the outcome of infection. A recent study showed that T-cells isolated and cultured in vitro from individuals with early stage chronic HCV infection (HCV non-resolvers) expressed higher levels of IL-10 compared with spontaneous HCV resolvers and our own observation corroborates these findings (Flynn et al., 2011). The increased IL-10 expression during chronic HCV infection suggests that ongoing viral replication either leads to higher IL-10 expression or elevated IL-10 is an underlying cause for the lack of viral clearance. IL-10 is classically considered an anti-inflammatory cytokine and stimulates an antibody-mediated Th2 response as opposed to the beneficial cellular-mediated Th1 response. However, in recent studies IL-10 has also been shown to possess immunosuppressive activity associated mainly with chronic viral infections such as HIV-1 and HCV (Brooks et al., 2006; Ejrnæs et al., 2006). Specifically, IL-10 overexpression was shown to lead to CD8⁺ T-cell exhaustion and the inability to mobilize T-cell-mediated clearance of persistent viruses (Accapezzato et al., 2004; Ejrnæs et al., 2006). In one report, serum IL-10 was detected in six of ten HBV-infected individuals during the first month post-exposure (Stacey et al., 2009). Dunn et al. (2009) studied immune responses during early acute HBV infection and detected inhibition of NK and T-cell responses coincident with induction of IL-10. However, in the case of HBV the immunosuppressive effect of IL-10 during acute infection is transient and does not preclude the ultimate viral control seen in the majority of individuals. A recent study examined serum cytokine expression in individuals with chronic HCV infection and detected elevated expression of IL-10 in individuals who did not clear the virus following treatment with peg-interferon and ribavirin (Yoneda et al., 2011b). This study indicated that higher IL-10 expression may be unfavourable to treatment-induced HCV clearance and it is possible this is the same for spontaneous HCV clearance. However, ours is the first report that shows significant serum IL-10 expression in the late post-ALT phase in individuals who progress to chronic HCV infection.

Our study has some limitations, including the small sample size, which allows for the relative determination of only large differences in cytokine, chemokine and growth factor concentration between groups. Another potential limitation is that the TTVS cohort includes only serum samples and not cells (whole blood or PBMCs) or tissue samples, which if available may have provided additional insights to host immune responses during acute HCV infection.

In summary, in HCV resolvers, serum IP-10 was significantly higher in the pre-ALT and early post-ALT phase compared with uninfected controls. We also observed significant increase in serum levels of IP-10, TNF-α and IL-10 in HCV non-resolvers compared with uninfected controls. Elevation of IP-10 and IL-10 coincided with the first significant ALT elevation and continued to remain elevated even in the late post-ALT phase in HCV non-resolvers. In addition to the lack of viral load decline within the first 16 weeks p.i. as a predictor of chronic HCV, serum levels of IP-10 and IL-10 should be evaluated further as possible biomarkers.

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References


