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# Delayed Induction, Not Impaired Recruitment of Specific CD8+ T Cells, Causes the Late Onset of Acute Hepatitis C

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## Abstract

**Background & Aims—**Hepatitis C virus (HCV) infection is characterized by lack of immune-mediated liver injury despite a high level of HCV replication during the incubation phase, which lasts about 8 weeks. We investigated whether this results from delayed recruitment of HCV-specific T cells and whether it facilitates HCV persistence.

**Methods—**Six chimpanzees were infected with HCV; blood and liver samples were collected for 28 weeks and analyzed for immune cells and chemokines.

**Results—**Two chimpanzees developed self-limited infections whereas the remaining 4 developed chronic infections. Levels of the chemokines CXCL10, CXCL11, CCL4, and CCL5 increased in blood and liver samples from all chimpanzees within 1 month of HCV infection. Chemokine induction correlated with intrahepatic type I interferon (IFN) responses in vivo and was blocked by neutralizing antibodies against IFN-β in vitro. Despite the early-stage induction of chemokines, the intrahepatic lymphocytic infiltrate started to increase no earlier than 8 weeks after HCV infection, when HCV-specific, tetramer+ CD8+ T cells appeared in the circulation. The HCV-specific CD8+ T cells expressed chemokine receptors when they were initially detected in blood samples, so they could be recruited to the liver as soon as they entered the circulation.

**Conclusions—**Chemokines are induced during early stages of HCV infection, which requires a type I IFN-mediated response. The delayed onset of acute hepatitis does not result from delayed



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recruitment of HCV-specific T cells, but could, instead be related to a primary delay in the induction of HCV-specific T cells. Divergent outcomes occur without evident differences in chemokine induction and T-cell recruitment.

### Keywords

liver disease; virology; immune response; monkey

## Introduction

Spontaneous resolution of the hepatitis C virus (HCV) infection depends on vigorous, early HCV-specific T-cell responses. Of special importance are CD8+ T cells, the main immune effector cells that recognize antigens in an MHC class I-restricted manner and lyse infected hepatocytes. HCV-specific memory CD8+ T cells persist in blood [1] and liver [2] for decades after HCV clearance and can mediate protective immunity [2-5]. Accordingly, experimental depletion of CD8+ T cells in chimpanzees delays HCV clearance until CD8+ T cells reappear [2].

A striking feature of HCV is its ability to establish persistence in the majority of infected persons [6]. This is associated with a relatively late appearance of HCV-specific T cells in the infected liver. Although HCV RNA titers increase to high levels in the circulation within days of infection [7, 8], it takes two to three months for HCV-specific T cell responses to become detectable in the liver [9]. This delayed intrahepatic appearance of HCV-specific T cells has been observed in assays in which T cells are subjected to in vitro expansion followed by functional assays [9] and in ex vivo molecular assays such as microarray [7, 10] and real-time PCR [8, 11]. It has therefore been suggested that the delayed recruitment of HCV-specific T cells may cause the high incidence of HCV persistence [8, 9].

The mechanisms resulting in recruitment of HCV-specific CD8 T cells to the liver in acute HCV infection are not fully understood. It has been suggested that incomplete differentiation and maturation of HCV-specific T cells [12] contribute to a defect in the expression of specific chemokine receptors on circulating HCV-specific T cells and thereby to delayed or impaired recruitment to the liver. It is also possible that there are defects in the specific intrahepatic events that are required for CD8 T cell recruitment, i.e. the induction of chemokines in the liver.

This study was designed to analyze the kinetics and mechanisms of CD8 T cell recruitment from the blood to the liver during the incubation phase and the acute phase of HCV infection. Acute hepatitis C was established in chimpanzees, the sole animal model of HCV infection, with an intravenously injected, monoclonal HCV strain. This unique approach resulted in consistent virological and clinical courses of infection, providing a well-controlled setting for the analysis of early immunological events. As intrahepatic factors, we studied the kinetics of chemokine and chemokine receptor expression. As extrahepatic factors relevant to CD8 T cell recuitment, we determined the frequency of HCV-specific CD8 T cells as well as the expression of chemokine receptors by these T cells throughout the course of infection. The chimpanzee model enabled us to study the kinetics of these factors in detail by frequent sampling of both blood and liver compartments. The results demonstrate that specific chemokines are induced by HCV RNA in a type I interferon (IFN)-mediated manner early and independent of the outcome of acute HCV infection. We propose that this delayed intrahepatic T cell response is not due to delayed recruitment but rather to delayed induction of HCV-specific CD8 T cells.

## Materials and Methods

### Chimpanzees

Six chimpanzees were housed under standard conditions for humane care in compliance with NIH guidelines at Association for Assessment and Accreditation of Animal Care accredited facilities. Study protocols were approved by the Public Health Service Interagency Model Committee (NIH) and the Animal Care and Use Committees of either the New Iberia Animal Care and Use Committee or the Center for Biologics Evaluation and Research. All chimpanzees were intravenously inoculated with 100 chimpanzee infectious dose 50 monoclonal (H77 p90, genotype 1a) HCV RNA-positive, anti-HCV-negative plasma [13]. HCV infection was self-limited in two (Ch6461 and Ch6455), and chronically evolving in four chimpanzees (Ch6475, Ch6411, ChA3A025 and Ch6412). The clinical and virological courses of HCV infection have previously been described for all chimpanzees except ChA3A025 [8]. Serum HCV RNA was quantified by real-time RT-PCR [8].

### Quantitation of chemokines

CXCL10 (IP-10), CCL4 (MIP-1β) and CCL5 (RANTES) were quantitated in plasma samples and cell culture supernatants using cytometric bead arrays (BD Biosciences, San Jose, CA) and a FACSCalibur flow cytometer (BD Biosciences). IFN-β, CXCL11 (I-TAC) and CXCL16 were quantitated using enzyme immunoassays (IFN EIA from Biosource International, chemokine EIAs from R&D Systems, Minneapolis, MN).

### Extraction of RNA, cDNA synthesis and real-time PCR

Total RNA was isolated from snap-frozen and mechanically homogenized liver biopsies and from Huh-7 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase digestion. Two hundred to four hundred nanogram RNA was reverse transcribed with the First-Strand cDNA Synthesis Kit (Marligen Biosciences, Ijamsville, MD). CXCL10, CXCL11, CCL4, CCL5, CXCL16, CD4, CD8β, CXCR3 and CCR5 mRNA were quantitated by real-time PCR with TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA). The amount of specific mRNA in HCV-infected liver biopsies or HCV-RNA transfected Huh-7 cells was calculated with Sequence Detector Software (version 1.6.3; Applied Biosystems), normalized to GAPDH and β-actin references and expressed as fold-increase over mRNA levels in pre-infection biopsies or mock-transfected Huh-7 cells, respectively.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded liver biopsies were sectioned at 4 microns, deparaffinized in xylene, re-hydrated in graded alcohol and rinsed in distilled water. Heat-induced epitope retrieval was achieved with 10mM Citrate buffer solution with 0.03% Tween 20, pH 6.0 in a pressure cooker for 4 minutes. Slides were cooled to room temperature for 30 min. Endogenous peroxidase activity was blocked with Dako Dual Endogenous Enzyme Blocker for 5 min, followed by Dako Serum-free Protein Bock for 10 min (Dako North America Inc., Carpinteria, CA). Polyclonal antibodies against either VCAM-1 or CXCL11 (both from Santa Cruz Biotechnology Inc, Santa Cruz, CA) were applied at 1:50 and 1:20 dilution, respectively, for 1 h at room temperature. Polyclonal anti-ICAM-1 (Sigma Prestige, St. Louis, MO) was applied at 1:25 dilution for 3 h at room temperature. A horseradish-peroxidase labeled goat anti-rabbit polymer (Dako, Carpinteria, CA) was in a 10-min detection step. All complexes were visualized with diaminobenzidene (Dako, Carpinteria, CA) for 10 min at room temperature. Slides were counterstained with methyl green (Vector, Burlingame, CA), dehydrated and mounted with permanent media.

### HCV RNA transfection of Huh-7 cells

Huh-7 hepatoma cells were grown in Dulbecco's Modification of Eagle's Medium containing 5% FCS, 4.5 g/l glucose, 50 μg/ml streptomycin, 50 IU/ml penicillin and 2 mM L-glutamine (complete DMEM). Huh-7 cells were transfected by DMRIE-C reagent (Invitrogen, Carlsbad, CA) in Opti-MEM medium (Invitrogen) using 2 μg proteinase K–treated H77 HCV RNA in vitro transcribed from pHCVH77 (kindly provided by J. Bukh, NIAID, USA) as previously reported [14]. After 4h culture medium was replaced by complete DMEM with or without 2,000 U/ml neutralizing anti-IFN-β (R&D Systems).

### HCV peptides

Six hundred pentadecamer peptides (Mimotopes, Clayton, Australia), overlapping by 10 amino acids and spanning the complete HCV genotype 1a (H77) sequence were resuspended at 20 mg/ml in dimethyl sulfoxide. Aliquots of each peptide suspension were pooled and diluted with PBS to obtain 49 mixes designed for a matrix array. Each peptide was present in 2 mixes, which were used in the initial antigen specificity screening of liver-infiltrating lymphocyte (LIL) lines in IFN-γ ELISpot assays. Single pentadecamer peptides were used in second round ELISpot assays, and single nonamer or octamer peptides were used in third round assays to define minimal T cell epitopes. The final concentration of each peptide was 1 μg/ml.

### Quantitation of liver infiltrating lymphocytes (LILs)

The liver biopsy size was measured in a sterile petri dish. Lymphocytes were isolated by mechanical homogenization, and counted in a hemocytometer under a light microscope.

### Identification of CD8 T cell epitopes and synthesis of Patr class I tetramers

LILs isolated from liver biopsies from up to 8 different time points per chimpanzee (months 6-12 after infection) were stimulated with anti-CD3 (30 ng/ml) and irradiated (3,000 rad) autologous peripheral blood mononuclear cells (PBMCs) in complete RPMI1640 (Mediatech) containing IL-2 (200 U/ml), IL-7 (10 ng/ml) and IL-15 (100 ng/ml, all from Peprotech, Rocky Hill, NJ). The cytokine-containing medium was replaced twice a week, and the cultures were re-stimulated 2-4 times at 3-week intervals with anti-CD3 and autologous PBMCs. To identify CD8 T cell epitopes, expanded LIL lines were subjected to IFN-γ ELISpot assays using either the matrix array or single peptides. To determine Patr class I restriction 100,000 cells from LIL lines were co-cultured with 20,000 HCV peptide-pulsed Patr-transfected 721.221 cells in 96-well U-bottom plates. The 48h-culture supernatant was tested for IFN-γ, TNF-α, GM-CSF and MIP-1β by cytometric bead array (BD Biosciences).

### Determination of Patr class I alleles, generation of Patr class I tetramers and multicolor flow cytometry

Patr-class I haplotypes were determined as described [15]. Patr class I tetramers (NIAID Tetramer Facility of the NIH AIDS Research and Reference Reagent Program.) were generated with the peptides that were identified with LILs. Ex vivo analysis of HCV-specific PBMC responses during the first 6 months of HCV-infection did not yield any additional Patr-tetramers of use in this study. Four million PBMCs were stained with ethidium monoazide (EMA) and subsequently with PE-conjugated Patr class I tetramer for 20 min at room temperature. Further staining was performed with anti-CD3-AlexaFluor700, anti-CCR5-APC-Cy7, anti-CD8-APC, anti-CD19-PeCy5 (all from BD Biosciences), anti-CXCR3-FITC (R&D Systems) and anti-CD14-PeCy5 (AbD Serotec, Raleigh, NC). To determine cut-off levels for CXCR3 and CCR5 expression, fluorescence-minus-one control samples were utilized. The stained cells were analyzed on an LSRII (BD Biosciences) using

FacsDiva Version version 4.1 (BD Biosciences) and FlowJo version 6.4.7 software (Tree Star, Ashland, OR). The frequency of tetramer$^+$ cells in the CD8$^+$ T cell population is reported as mean and standard deviation of triplicate samples.

## Results

### Specific chemokines appear in the plasma in the early stage of acute HCV infection

Acute HCV infection was self-limited in chimpanzees Ch6461 and Ch6455 and chronically evolving in chimpanzees Ch6475, Ch6411, ChA3A025 and Ch6412. Serum alanine aminotransferase (ALT) levels and HCV RNA titers during the first 6 months of infection have previously been reported for all chimpanzees except ChA3A025 [8] and are displayed in Fig. 1A for ease of reference. In chimpanzees with a self-limited course of hepatitis, the serum ALT peak was followed by HCV clearance (Fig. 1A). In chimpanzees with a chronically evolving course of hepatitis, the serum ALT peak was followed by a 2-3 $\log_{10}$ (i.e. > 99%) decrease of serum HCV RNA titer of about $10^4$-$10^5$ HCV genome copies per ml serum in the chronic phase. One of the four chronic chimpanzees (Ch6412) controlled HCV RNA temporarily at levels undetectable in the serum (week 19 to 23) until consistent HCV RNA titers of $10^4$-$10^5$ genome copies per ml were established and maintained for at least one year of follow up (Fig. 1A, and not shown).

To determine which chemokines were produced during the course of HCV infection, concentrations of CXCL10 and CXCL11 (which both bind to the CXCR3), CCL4 and CCL5 (which both bind to CCR5) and CXCL16 (which binds to CXCR6) were tested in serial plasma samples. All chemokines are known to mediate T cell recruitment to peripheral inflammatory tissues. As shown in Fig. 1B-E CXCL10, CXCL11, CCL4 and CCL5 concentrations started to increase within a month after HCV infection and often reached their peak level at the time of maximum liver injury (ALT peak). In contrast, CXCL16 levels did not increase throughout the entire course of acute HCV infection (Fig. 1F). The onset and kinetics of chemokine release into the circulation did not predict the outcome of acute HCV infection.

### Induction of chemokine mRNA in the liver correlates to the intrahepatic type I IFN response

To study intrahepatic events relevant to lymphocyte recruitment chemokine and adhesion molecule expression were determined in serial liver biopsies by real-time PCR (Fig. 2) and/or immunohistochemistry (Fig. 3). Intrahepatic CXCL10, CXCL11, CCL4 and CCL5 mRNA levels increased early after infection and maximal expression coincided with the ALT peak (Fig. 2). Notably, CXCL16 mRNA levels remained much lower than those of the other chemokines throughout of the course of infection (Fig. 2E). Consistent with the results from the plasma analysis the kinetics of chemokine expression in the liver did not predict the outcome of acute HCV infection.

Chemokine expression was not limited to hepatocytes but also found on sinusoidal cells as early as 4 weeks after HCV infection, as demonstrated for CXCL11 by immunohistochemistry (Fig. 3). Adhesion molecules ICAM-1 and VCAM-1 which facilitate T cell entry into the liver are detectable at the same time (Fig. 3). Expression of a second adhesion molecule, VCAM-1, was slightly increased in sinusoidal cells at the same time.

Next, intrahepatic chemokine expression was compared to the intrahepatic IFN response. Although CXCL10 and CXCL11 are known as classical IFN-γ-induced chemokines [16] the increase in their intrahepatic mRNA levels occurred weeks prior to the increase of IFN-γ and CD8 mRNA levels that we previously reported [14] suggesting that it was not induced by the adaptive immune response. Instead, the induction of chemokine mRNA correlated to the

intrahepatic type I IFN response, as exemplified by mRNA levels of the type I IFN-induced enzyme 2,5-oligoadenylate synthetase-1 [2,5-OAS-1; reported for all chimpanzees except ChA03A025 in [14] and shown for references purposes in Fig. 2F]. These temporal correlations suggest a role of type I IFNs rather than IFN-γ in the induction of CXCR3- and CCR5-binding chemokines in the early phase of HCV infection.

### Intracellular HCV RNA induces selected chemokines in a type I IFNs-dependent manner

To elucidate the mechanisms of chemokine induction Huh-7 hepatoma cells were transfected with H77 HCV RNA. As shown in Fig. 4A transfection of Huh7 cells with full-length HCV RNA induced IFN-β at mRNA and protein levels. CXCL10, CXCL11, CCL4 and CCL5, but not CXCL16 were induced simultaneously (Fig. 4B). Antibody-mediated neutralization of IFN-β abrogated the secretion of chemokines (Fig. 4C). Collectively, these data demonstrate that intracellular HCV RNA induces the production of chemokines in a type I IFN-dependent manner.

### Intrahepatic increase in CXCR3 and CCR5 mRNA levels coincides with intrahepatic increase in CD8β mRNA levels

To investigate how intrahepatic chemokine induction elated to T cell recruitment, lymphocytes were isolated and counted from serial liver biopsies. The number of intrahepatic lymphocytes started to increase 8-12 weeks after HCV infection (Fig. 5A) in parallel to the increase in serum ALT levels (Fig. 1A). CD8β but not CD4 mRNA levels increased simultaneously (Fig. 5B). These results suggest that liver-infiltrating CD8 rather than CD4 T cells contributed to the ALT peak and to the subsequent reduction in HCV RNA titer.

Because the mRNA level of chemokines increased in the acutely infected liver (Fig. 2) we wondered whether mRNA levels of their receptors CXCR3 and CCR5 changed accordingly as a result of T cell recruitment. CXCR3 and CCR5 are typically upregulated on activated, differentiated effector T cells and determine the homing of these cells to peripheral inflammatory tissues. Indeed, intrahepatic CXCR3 and CCR5 mRNA levels increased in parallel to the observed increase in CD8β mRNA level (Fig. 5C and D). Collectively, these data suggest that recruitment of $CXCR3^+$ and $CCR5^+$ CD8 T cells is closely correlated to the expression of chemokines.

### HCV-specific CD8 T cells are recruited to the liver as soon as they appear in the circulation

All data shown so far relate to the total population of T cells, irrespective of their antigen-specificity. To study HCV-specific T cells we Patr-typed the chimpanzees, tested liver-infiltrating T cells for antigen-specificity and Patr-restriction, and generated Patr class I tetramers. Several chimpanzees mounted CD8 T cell responses against identical HCV peptides and were therefore studied with the same Patr-tetramers (Suppl. Table 1). Representative FACS data are shown in Fig. 6A and Suppl. Fig. 1 and all results are summarized in Fig. 6B. HCV-specific, $tetramer^+$ CD8 T cells became detectable in the blood approximately 8-12 weeks after infection, with peak 11-15 weeks after infection (Fig. 6A and B). About 40% of $tetramer^+$ CD8 T cells expressed CXCR3 and/or CCR5 after they appeared in the circulation (week 8 in Ch6461 and week 13 in Ch6411, Suppl. Fig. 2A and B). CXCR3 and CCR5 were preferentially expressed by $tetramer^+$ CD8 T cells when compared to the total CD8 T cell population (Suppl. Fig. 2A). After the initial peak in frequency, the percentage of $CXCR3^+$ and/or $CCR5^+$ cells within the $tetramer^+$ CD8 T cell population decreased (Suppl. Fig. 2B). These data suggest that a subset of $CXCR3^+$ and/or $CCR5^+$ cells was selectively recruited from the circulating $tetramer^+$ T cell population. Indeed, the appearance of HCV-specific, $CXCR3^+$ and $CCR5^+$ CD8 T cells in the blood coincided precisely with the increase in CD8β (Fig. 5B), CXCR3 (Fig. 5C) and CCR5

mRNA levels (Fig. 5D) in the liver. These data suggest that a proportion of HCV-specific CD8 T cells were recruited into the liver as soon as they appeared in the circulation. Furthermore, the correlation between the increase in CD8β mRNA levels and the previously reported intrahepatic IFN-γ mRNA levels [14] suggests that these cells exert immediate effector function upon recognition of their cognate antigen in the liver.

## Discussion

This study was designed to analyze timing and mechanisms of CD8 T cell recruitment to the liver in acute HCV infection. The prospective *in vivo* data indicate a role for CXCL10, CXCL11, CCL4 and CCL5, but not CXCL16 in CD8 T cell recruitment, and a role for type I IFNs in the induction of these chemokines. The latter was confirmed by *in vitro* experiments in which intracellular HCV RNA induced the expression of CXCL10, CXCL11, CCL4 and CCL5 in human hepatoma cells in a type I IFN-dependent manner. Thus, HCV RNA-induced type I IFN recruits CD8 T cells to the liver via induction of chemokines.

Based on our findings, we propose the following sequence of events in acute HCV infection (Fig. 7). The earliest detectable event is an intrahepatic type I IFN induction in response to intracellular HCV RNA. The source of type I IFN is currently undefined *in vivo*, but *in vitro* experiments suggest that it may be produced by either hepatocytes (this study and [14] or plamacytoid dendritic cells [17]). This is followed by production of chemokines 2-8 weeks after HCV infection. Chemokine expression is not limited to hepatocytes but also found on sinusoidal cells, as demonstrated for CXCL11 (Fig. 3) with the caveat that we did not have established immunohistochemistry techniques for the other chemokines that were quantitated by realtime PCR in the liver and EIA in blood. Adhesion molecules ICAM-1 and VCAM-1 which facilitate T cell entry into the liver are detectable at the same time (Fig. 3). However, HCV-specific tetramer$^+$ T cells become detectable in the circulation not earlier than 8-12 weeks after infection. They express CXCR3 and/or CCR5 and are immediately recruited into the liver as evidenced by the selective disappearance of CXCR3$^+$ and/or CCR5$^+$ cells from circulation. Amplification of the intrahepatic lymphocytic infiltrate results in liver injury, as evidenced by serum ALT elevation, which is followed by a decrease of HCV RNA titer and/or HCV clearance.

This specific sequence of events that we observed in HCV infection differs from that in other hepatotropic viral infections. In acute hepatitis B virus (HBV) infection, for example, intrahepatic type I IFN responses appear to be absent as determined by a lack of detectable 2,5-OAS-1 mRNA induction [18]. Accordingly, there is no increase in the expression of CXCL10 and CXCL11 during the incubation phase of HBV infection [18]. CXCL10 and CXCL11 are only induced in the later, acute phase of HBV infection, concomitant to an increase in intrahepatic IFN-γ mRNA [18], which is a well known inducer of CXCL10 and CXCL11. These results suggest that the mechanisms of chemokine induction and T cell recruitment differ in HCV and HBV infection.

Because the generation of Patr-tetramers required the mapping of T cell epitopes for the each chimpanzee, these reagents were not yet available at the time the liver biopsies were obtained. Therefore, our study had to rely on molecular methods to quantitate T cell subsets and chemokine receptor expression and it was not possible to differentiate between HCV-specific T cells and non-specific bystander cells in the liver. Nevertheless, several observations support the notion that the early intrahepatic immune response was antigen-specific. First, the population of HCV-specific, tetramer$^+$ CD8 T cells that expressed CXCR3 and/or CCR5 selectively decreased in the blood at the same time as CD8β mRNA levels increased in the liver, suggesting recruitment of HCV-specific, tetramer$^+$ CD8 T cells

from the circulation to the liver. Second, adoptive transfer studies demonstrated in a mouse model of acute hepatitis B that virus-specific T cells are the initiators of a cascade that leads to amplification of the intrahepatic infiltrate by non-specific bystander cells and ultimately, to increase of serum ALT levels and acute hepatitis [19].

Chemokine-dependent mechanisms of T cell recruitment also operate in chronic HCV infection as shown in several cross-sectional studies that examined the expression of chemokine receptors on liver-infiltrating T cells [20, 21], and intrahepatic expression of chemokines [20, 22]. Although these cross-sectional studies did not establish a temporal relation between the expression of chemokine receptors on circulating T cells, the induction of chemokines in the liver and the recruitment of T cells to the liver, they implicate that CXCR3 [20-22], CCR5 [21], CCR7 [23], CXCR6 [21], and CXCR4-binding chemokines [24] contribute to chronic HCV infection. Our study demonstrates that CCR5- and CXCR3-binding chemokines but not the CXCR6-binding chemokine CCL16 play a role in acute HCV infection. The continuing induction of CCR5-binding rather than the CXCR3-binding chemokines 6 months after infection in those animals that progress to chronic HCV infection suggests a shift in intrahepatic chemokine production as acute hepatitis turns into chronic hepatitis.

In this context it is of interest that an antagonist to CXCL10 has recently been proposed to contribute to nonresponse to interferon-based therapy in chronic hepatitis C [25]. We do not think that this mechanism explains our findings for the following reasons: First, the current study relates to the spontaneous outcome of acute HCV infection not to treatment-induced outcome of chronic HCV infection. Second, CXCL10 levels do not predict outcome of acute HCV infection whereas they are negative predictors of treatment outcome [25]. Third, the late appearance of HCV-specific T cells in the circulation in acute hepatitis C in the current study coincides precisely with the onset of the intrahepatic CD8 T cell infiltrate suggesting impairment of T cell priming rather than recruitment.

Importantly, different outcomes of HCV infection were observed even though the induction of HCV-specific T cells, the expression of chemokine receptors on HCV-specific T cells, the expression of chemokines in the liver and the recruitment of CD8 T cells to the liver occurred at comparable time points in all chimpanzees. These results differ from those of a previous study, which suggested an association between impaired T cell recruitment to the liver and a chronic course of HCV infection [9]. This study, however, tracked the appearance of HCV-specific T cells in the blood with in vitro assays that required several weeks of T cell expansion rather than using an ex vivo analysis with tetramers. This methodical difference is important because HCV-specific CD8 T cells have been described as being “stunned” with impaired proliferative capacity [9, 26] and thus might not have been detected by functional assays. Other differences between the two studies are the nature of the HCV inoculum and the route of HCV infection. Chimpanzees were inoculated with different HCV strains via either an intrahepatic or intravenous route of infection in the previous study [9], and it is possible that the heterogeneity in the inoculum and the route of infection altered T cell priming and/or their recruitment to the liver. It is therefore an important finding of our study that even HCV infections where viral factors are kept uniform and where the timing of induction and recruitment of HCV-specific T cells do not vary may result in divergent outcomes, i.e. in HCV clearance or persistence. Future studies should therefore address whether host factors that influence antigen presentation and recognition in the HCV-infected liver contribute to the divergent outcomes of HCV infection.

Intriguingly, defects in antigen presentation may also contribute to the late induction of HCV-specific immune responses that we propose in this study (Figure 8). It is known that the effectiveness of antigen-transport to and T cell priming in perihepatic lymph nodes

depends on the number and viability of infected cells and the inflammatory environment. Cell death is known to enhance the immunogenicity of cell-associated viral antigens via the crosspriming pathway [27] HCV may neither kill enough hepatocytes to release antigen nor cause sufficient inflammation to induce crosspriming. In addition, a strong natural killer cell response, resulting from the HCV RNA-induced type I IFN reported in this study, may reduce the number of antigen expressing dendritic cells in a perforin-mediated manner [28]. If the dendritic cell-mediated transport of HCV antigens from the liver to the lymph nodes was reduced the priming of effective T cell responses and the appearance of HCV-specific T cells in the circulation and the liver would be delayed as observed in this study. Thus, we propose that the delayed appearance of HCV-specific CD8 cells in the liver during acute HCV infection is due to a delayed induction rather than delayed recruitment of HCV-specific T cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
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| **ALT** | alanine aminotransferase |
| **EIA** | enzyme immunoassay |
| **EMA** | ethidium monoazide |
| **HCV** | hepatitis C virus |
| **IFN** | interferon |
| **LILs** | liver-infiltrating lymphocytes |
| **2,5-OAS-1** | 2,5-oligoadenylate synthetase-1 |
| **PBMCs** | peripheral blood mononuclear cells |

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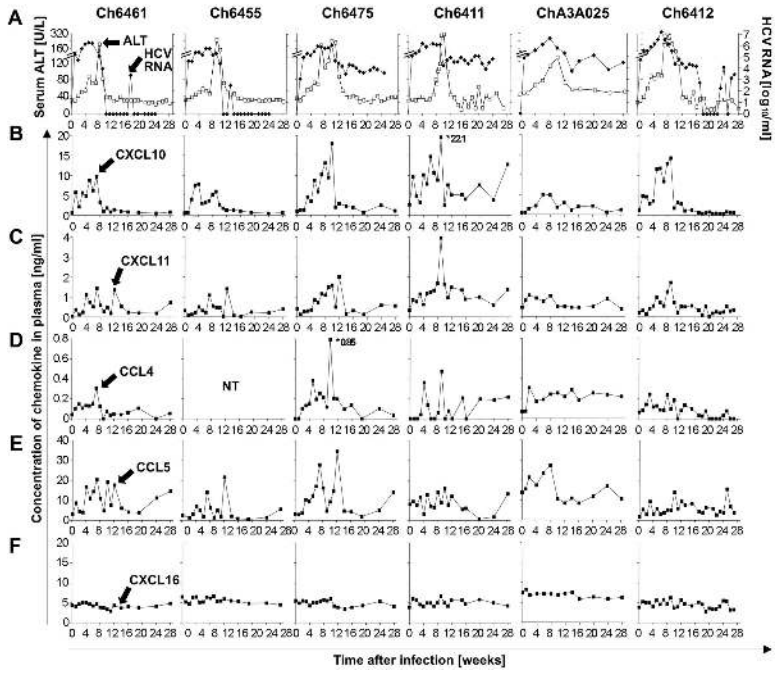


**Fig. 1. Specific chemokines increase in the circulation in the early stage of acute HCV infection**

**(A)** Serum ALT levels (open squares) and serum HCV RNA titers (black diamonds) during the first 6 months after HCV infection have previously been reported for all chimpanzees except ChA3A025 [8, 14, 29] and are presented for ease of reference. **(B-E)** Plasma CXCL10 **(B)**, CXCL11 **(C)**, CCL4 **(D)** and CCL5 levels **(E)** increase during acute HCV infection but CXCL16 levels **(F)** do not. NT, not tested. * The number next to the asterisk specifies a value off scale.

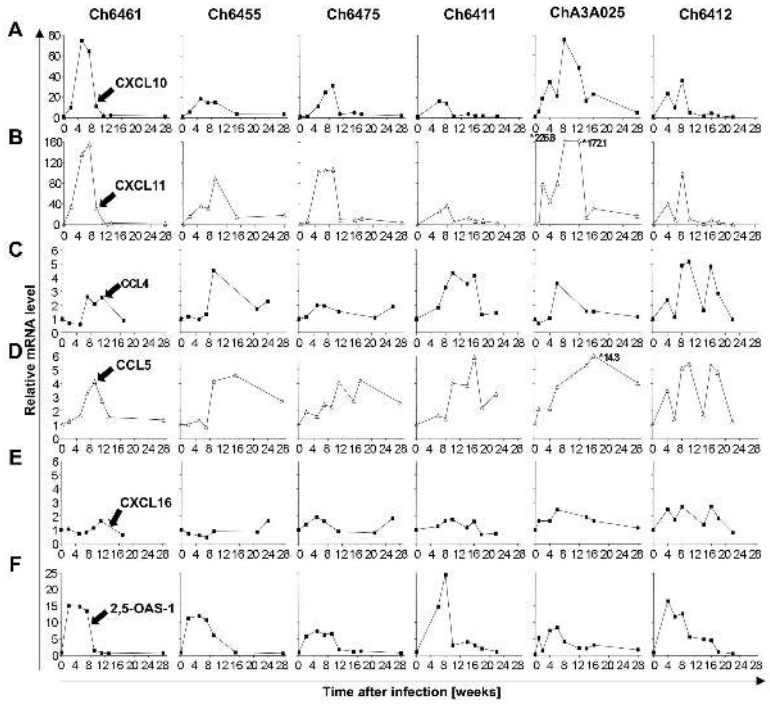


**Fig. 2. Induction of chemokine mRNA in the liver correlates to the intrahepatic type I IFN response**

**(A-E)** Serial liver biopsies were analyzed for CXCL10 (**A**), CXCL11 **(B)**, CCL4 (**C**), CCL5 **(D)**, and CXCL16 **(E)**. **(F)** 2,5-OAS-1 mRNA levels for all chimpanzees except ChA3A025 [14] are presented for ease of reference. mRNA levels were normalized to endogenous references (GAPDH and β–actin) and expressed as fold increase over preinfection levels. * The number next to the asterisk specifies a value off scale.

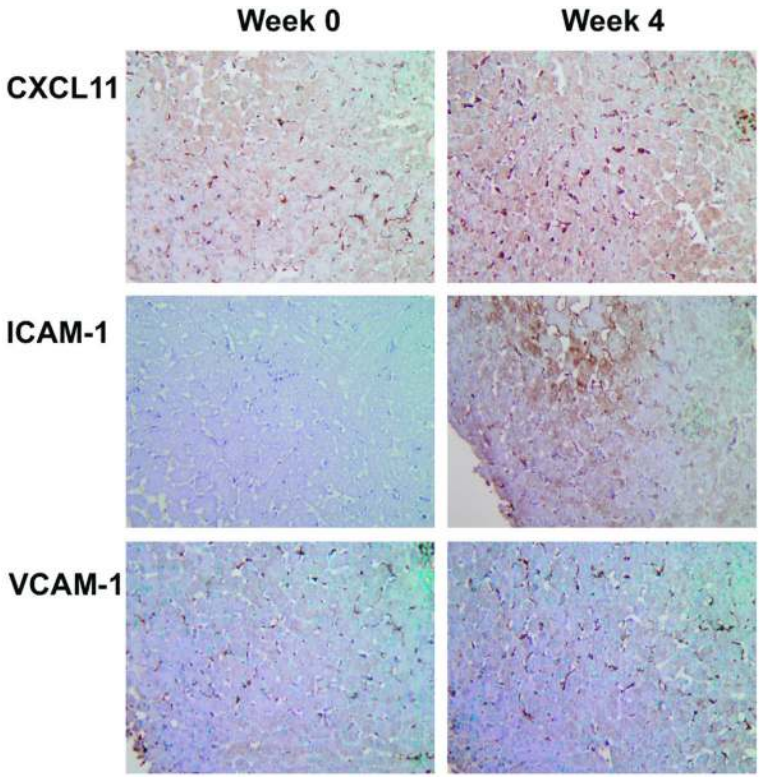


**Fig. 3. Intrahepatic CXCL11, ICAM-1 and VCAM-1 expression during acute hepatitis C**
Liver biopsies taken at weeks 0 and 4 of HCV infection from chimpanzee A03A025 were stained for CXCL11, VCAM-1 and ICAM-1 (original magnification x 200).

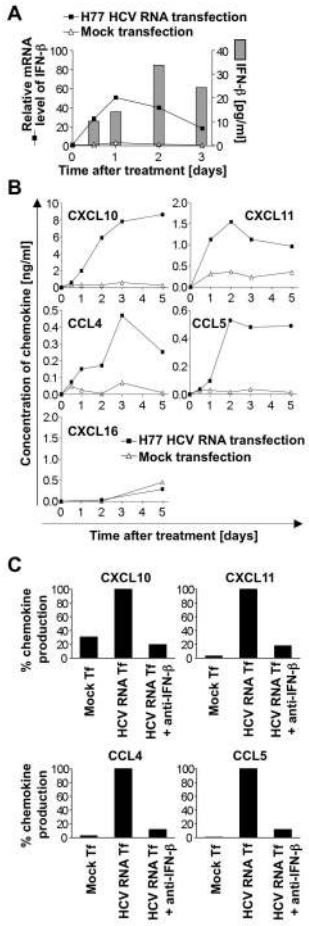


**Fig. 4. HCV RNA induces selected chemokines in a type I IFN dependent manner**

Huh-7 hepatoma cells were H77 HCV RNA (black squares) or mock transfected (open triangles). **(A)** IFN-β mRNA was determined by real-time PCR. Secreted IFN-β was detectable by EIA in supernatants of HCV RNA-transfected, but not mock-transfected Huh-7 cells (not shown). **(B)** The concentration of chemokines secreted by H77 HCV RNA (black squares) or mock transfected (open triangles) Huh-7 hepatoma cells, was determined by cytometric bead array or EIA. **(C)** Blocking of IFN-β with neutralizing antibodies decreased the level of secreted chemokines.

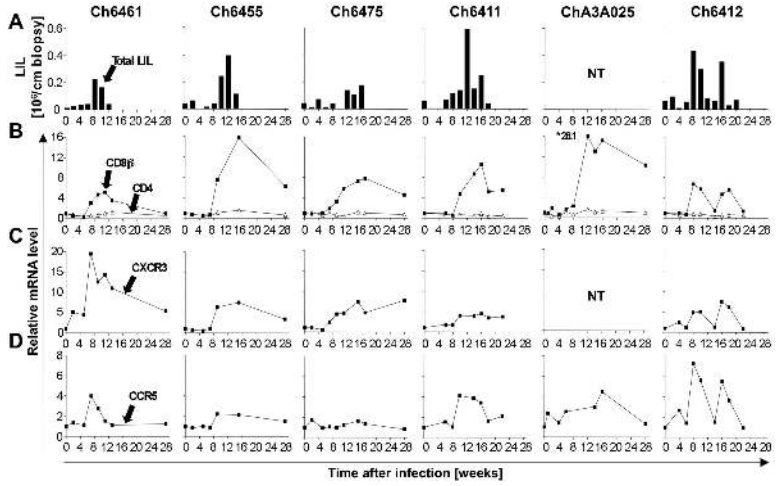


**Fig. 5. Intrahepatic increase in CXCR3 and CCR5 mRNA levels coincides with accumulation of lymphocytes in the liver and increase in CD8β mRNA levels**

Serial liver biopsies were analyzed for **(A)** the number of liver infiltrating lymphocytes per cm biopsy, **(B)** CD8β (black squares) and CD4 (open triangles), **(C)** CXCR3 and **(D)** CCR5 mRNA levels. CD8β mRNA levels have previously been reported for all chimpanzees except ChA3A025 [14] and are presented for ease of reference. NT, not tested. * The number next to the asterisk specifies a value off scale.

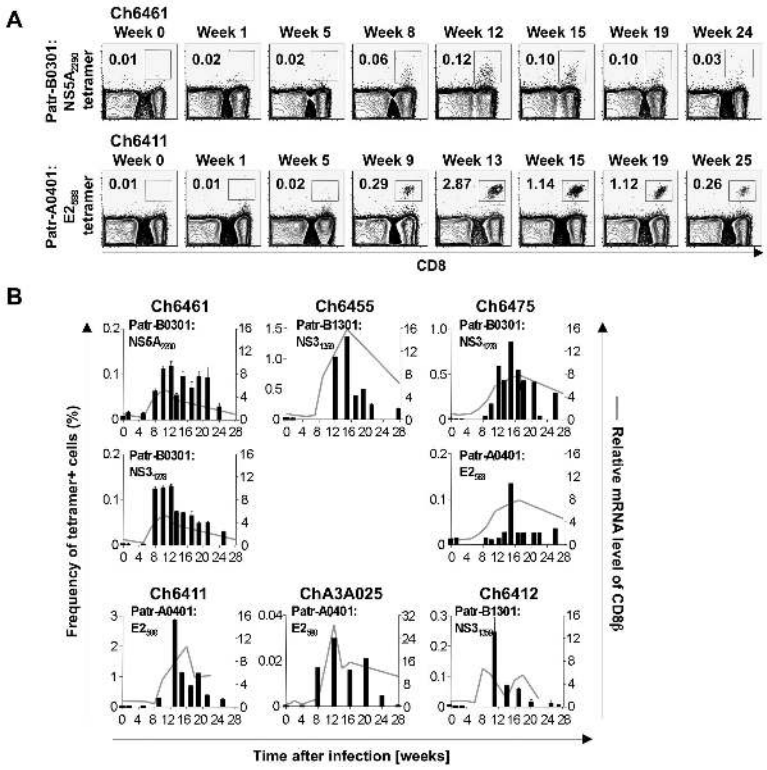


**Fig. 6. HCV-specific CD8 T cells are recruited to the liver as soon as they appear in the circulation**

**(A)** Representative FACS dot plots show the percentage of tetramer$^+$ CD8 T cells. **(B)** Black bars represent percentage of tetramer$^+$ T cells in the CD8 T cell population (mean and standard deviation from 3 independent experiments). The grey line represents intrahepatic CD8β mRNA levels.

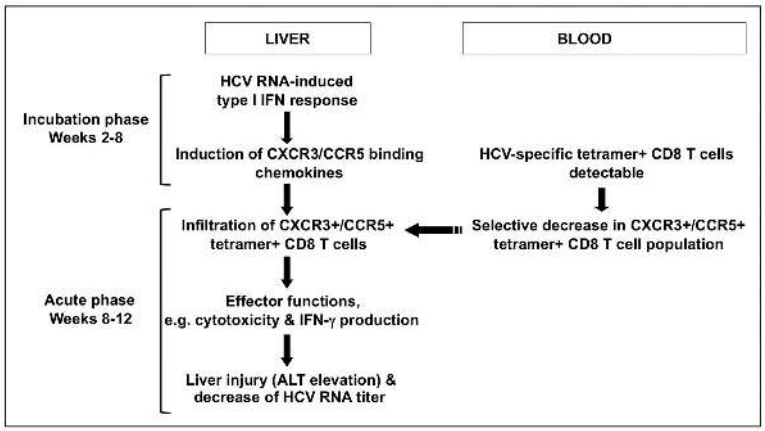


**Fig. 7. Model of the sequence of events in liver and blood culminating in the acute phase of hepatitis C**

See text for details.