Decrease in hepatic CD56<sup>+</sup> T cells and Vα24<sup>+</sup> natural killer T cells in chronic hepatitis C viral infection

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**Background/Aims:** The intrahepatic immune system is likely to play a key role in determining the outcome of hepatitis C virus (HCV) infection. The hepatic lymphocyte repertoire is characterised by high CD8/CD4 T cell ratios and large numbers of γδ T cells, natural killer (NK) cells, NK T cells and NK receptor-positive T cells. It is not known which of these populations contribute to immunity against HCV or immune pathology.

**Methods:** To explore the relative contributions of lymphocyte subpopulations, we have compared the intrahepatic lymphocyte repertoires and cytokine expression in 13 patients with mild chronic hepatitis C infection, 14 with end-stage hepatitis C cirrhosis and five histologically normal livers by flow cytometry and immunohistochemistry.

**Results:** CD4<sup>+</sup> T cells bearing αβ T cell receptors (TCR) were significantly expanded in livers with chronic HCV infection while CD56<sup>+</sup> αβ T cells and Vα24 TCR-positive T cells were significantly depleted. Expanded CD4<sup>+</sup> T cells were predominantly Th1 cells, producing interferon-γ but not interleukin-4.

**Conclusions:** Failure to resolve HCV infection may be due to deficient innate and/or memory immune responses, while Th1 cells may mediate immune pathology.

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**Keywords:** Natural killer cells; Hepatitis C virus; T cell receptor

1. Introduction

Hepatitis C virus (HCV) infects over 170 million people worldwide [1]. A minority (~20%) resolve acute hepatitis and clear the virus, but most develop life-long infection, making HCV a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma [1,2]. The host immune response to HCV antigens is thought to determine whether viral clearance or chronicity occurs. Studies in chimpanzees (the only animal model for HCV infection) suggest that resolution or persistence of HCV infection depends on the strength of intrahepatic lymphocyte responses that are generated at the early stages of acute hepatitis [4]. Analyses of peripheral HCV-specific T cell responses in humans support this notion [5]. In contrast, chronicity of HCV infection is associated with weaker early HCV-specific T cell responses and a persistence of responses that contribute to liver damage [3–5].

The intrahepatic immune system is characterised by a unique repertoire of lymphocytes [6,7]. In addition to conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells, the liver contains large numbers of natural killer (NK) cells,
γδ T cells and T cells that express NK cell stimulatory, costimulatory and inhibitory receptors (NKR)s that appear to have important innate and regulatory functions in hepatic immunity. Hepatic NKR-expressing T cells include both conventional major histocompatibility complex (MHC)-restricted T cells that recognise peptide antigens [8] and NKT cells which recognize glycolipid antigens presented by the non-classical antigen presenting molecule CD1 [9–11]. The latter cell type frequently expresses invariant T cell receptor (TCR) α-chains (Vα24 JαQ in humans and Vα14 Jα281 in mice) [12,13].

Most work on immunity against HCV has focused on the generation of hepatic HCV-specific T cell lines in vitro [4,5,14,15], but recent studies have highlighted essential roles for NK cells, γδ T cells and NKR− T cells in immunity against hepatotropic viruses and in immunopathology. Hepatic NK cells and γδ T cells are necessary for optimal priming and cytolytic function of virus-specific T cells via the production of interferon-γ (IFN-γ), but can also directly induce apoptosis of virus-infected hepatocytes [10,16,17]. NK cell activity can be directly modulated by HCV [18,19]. NKR− T cells can control virus-specific T cell differentiation and NK cell activation and they expand in response to viruses in the liver and other organs [8,20,21]. CD1-restricted NKT cells have also been implicated in effective immunity against hepatitis B virus (HBV) [10]. It is not known which lymphocyte populations contribute to or control liver damage in HCV infection. We have therefore compared the intrahepatic lymphocyte repertoires in 13 patients with mild chronic HCV infection, 14 with end-stage HCV cirrhosis and 5 healthy livers in order to identify putative lymphocyte populations that influence resolution or progression of HCV-mediated liver disease.

2. Materials and methods

2.1. Patients and controls

Liver specimens were obtained from percutaneous needle biopsy tissue taken for routine histological examination from 14 patients (mean age 40.9, range 24–60, seven males and seven females) attending St. Vincent’s University Hospital (SVUH), Dublin. All had circulating anti-HCV antibodies, HCV RNA, and histological evidence of chronic hepatitis consistent with HCV disease. The average duration of HCV infection was 17 years. Eight patients were infected with HCV genotype 1, five with genotype 3, one with genotype 2 and the genotype was unknown in one. Thirteen patients had non-cirrhotic HCV liver disease as determined by a histological stage of less than four using the modified HAI scoring system [22] and one had HCV cirrhosis. Samples of explant liver from a further 13 patients (mean age 50; range 30–62, nine males and four females) who were undergoing liver transplantation for end-stage HCV cirrhosis at SVUH and King’s Healthcare (KH), London, were also studied. Of these, eight, one and two were infected with HCV genotypes 1, 2 and 3, respectively. The HCV genotype was not determined for two of these patients. Histological examination of wedge biopsy samples showed that all had established cirrhosis consistent with HCV infection. No patient had evidence of concurrent HBV infection, alcohol-induced or autoimmune liver disease. Histologically normal liver specimens were obtained from five donor organs (mean age 42, range 36–62; all males) taken at the time of liver transplanta-

2.2. Isolation of hepatic HMC

Liver biopsy specimens were immediately placed in complete RPMI medium (Gibco BRL, Paisley, UK) for transport. Donor organs were extensively perfused with University of Wisconsin solution prior to obtaining the biopsy. Needle and explant biopsies were washed three times in 50 ml salt-free Hank’s Balanced Salts Solution (Gibco-BRL) to remove residual blood. Hepatic mononuclear cells (MNC) were isolated from donor and HCV explant liver biopsies as described previously [23]. A modification of this procedure was used to extract hepatic MNC from needle biopsy specimens. Briefly, 0.025% collagenase IV (Sigma–Aldrich, Ireland) was used in the disruption enzyme mixture. Mechanical disruption was carried out using a glass pestle. The final cell pellet was resuspended in 300–500 μl RPMI. These procedures produced optimal yields of viable cells from the small amount of tissue available from needle biopsy material (data not shown).

2.3. Immunohistochemical analyses

Liver biopsy tissue was mounted in cryopreservative embedding medium (OCT, Tissue Tek, Finetek Europe, B.V., The Netherlands) before being transferred to liquid nitrogen for subsequent fluorescent and immunoperoxidase immunohistochemical studies. Fluorescent microscopy was performed on a Nikon TE300 microscope equipped with a Leica DC100 digital camera. The following primary antibodies were used: anti-CD3 rabbit anti-human polyclonal (Dako, Ely, UK; 1/10) and monoclonal anti-CD4 clone HT310 (IgG1, Dako, 1/50). Secondary antibodies consisted of Rhodamine-labelled anti-rabbit Ig (Jackson Laboratories, 1/40) and FITC-labelled anti-mouse IgG1 (Southern Biotechnology, AL, USA, 1/20). Immunoperoxidase staining of frozen sections was carried out using the Vectastain® Elite ABC Kit (Vector Laboratories, CA, USA) according to the manufacturers instructions. Staining for interferon-γ (IFN-γ) was performed using mAb clone 25718.11 (R&D Systems, Abingdon, UK, 1/10).

2.4. Antibodies and flow cytometry

mAb specific for CD3, CD4, CD8, CD19, CD56, γδ TCR, γδ TCR, IFN-γ, interleukin-4 (IL-4) and isotype-matched controls were obtained from Becton Dickinson (Oxford, UK). The mAb specific for the Vα24 TCR α-chain (clone C15) was obtained from Coulter Immunotech (Marseille, France). Phenotypic analysis of hepatic lymphocytes was performed by mAb staining of hepatic MNC and analysis on a FACScan flow cytometer with CellQuest software (Becton Dickinson). The relative proportions of lymphocyte subpopulations were determined as percentages of the total numbers of cells in a lymphogate defined by forward and side scatter properties, or as percentages of CD3+ cells.

2.5. Analysis of cytokine production

Hepatic MNC were stimulated in vitro for 6 h with phorbol myristate acetate (PMA) and ionomycin or plate-bound anti-CD3 mAb (clone HIT3a; PharMingen, Oxford, UK) in the presence of brefeldin A (Sigma–Aldrich). Cytokine production by CD4+ hepatic T cells was examined by a combination of cell-surface and intracytoplasmic mAb staining for IFN-γ and IL-4 and analysed by flow cytometry as described previously [24].

2.6. Statistical analyses

The Mann–Whitney U test was used to compare differences between cell populations and patient/control groups and a P value of less than 0.05 was taken as significant.
3. Results

3.1. Intrahepatic MNC yields

Histologically normal liver specimens contained \(0.75 - 4.3 \times 10^3\) cells per milligram of tissue (mean \(1.75 \times 10^3\)). Yields were increased from liver biopsies from patients with non-cirrhotic HCV infection (mean \(2.12 \times 10^3\) MNC/mg, range \(1.0 - 3.9 \times 10^3\) MNC/mg) and cirrhotic HCV disease (\(3.98 \times 10^3\) MNC/mg, \(2.6 - 5.5 \times 10^3\) MNC/mg).

3.2. Depletion of intrahepatic CD56^+ and expansion of CD56^- T cells in chronic HCV infection

Flow cytometric analysis of freshly isolated hepatic MNC showed that the proportions of conventional CD3^+ CD56^- T cells were significantly higher in patients with non-cirrhotic HCV infection (median 68.2%, range 24.7–92.6%) than in histologically normal livers (42.8%, range 25.5–57.3%; \(P = 0.009\); Fig. 1). These percentages fell slightly in patients with end-stage HCV cirrhosis. In contrast, the proportions of NKR^+ T cells expressing CD56 were significantly lower in patients with non-cirrhotic HCV disease (median 4.6%, range 1.1–23.3%; \(P = 0.005\); Fig. 1) and cirrhotic liver disease (5.8%, 0.2–13.7%; \(P = 0.002\)) compared to controls (17.1%, 10.7–28.1%). The proportions of CD3^-CD56^- NK cells were reduced among hepatic MNC from patients with non-cirrhotic and cirrhotic chronic HCV disease compared to normal controls, however, these reductions did not reach statistical significance (Fig. 1). B cell (CD19^+) percentages were similar in hepatic MNC taken from normal livers and non-cirrhotic and cirrhotic HCV-infected livers (Fig. 1).

3.3. Expansion of intrahepatic CD4^+ and depletion of CD8^+ and CD4^-CD8^- T cells in chronic HCV infection

Among CD3^+ T cells, CD4^+ cell percentages increased from a median of 17.4% (9.3–20.4%) to 30.2% (11.8–33.1%) in patients with mild chronic HCV disease (\(P = \)...
and to 43.7% (11.6–53.2%) in patients with severe/
end-stage HCV disease ($P = 0.01$) (Fig. 2A, B). This
increase was associated with a concomitant decrease in
CD8$^+$ T cells in cirrhotic (45.2%, 26.9–67.2%; $P = 0.04$)
but not in non-cirrhotic HCV-infected livers when
compared to normal hepatic MNC populations (69.6%;
62.1–74.6%). Double-negative (DN) CD4$^-$CD8$^-$ cells
were depleted in cirrhotic (4.4%, 1.5–13.2%; $P = 0.05$),
but not in non-cirrhotic HCV-infected livers compared to
normal controls (10.0%, 2.3–23.6%), while double-positive
(DP) CD4$^+$CD8$^+$ T cells were unchanged (Fig. 2A, B).
Two-colour immunohistochemical analysis of four frozen

Fig. 2. Intrahepatic CD4$^+$ T cells are expanded and CD8$^+$ and double-negative CD4$^-$CD8$^-$ T cells are depleted in chronic HCV infection. (A) Distribution of T cells bearing CD4$^+$, CD8$^+$, CD4$^-$CD8$^-$ and CD4$^+$CD8$^+$ phenotypes among hepatic MNC isolated from histologically normal donor liver specimens and liver specimens taken from patients with non-cirrhotic or cirrhotic chronic HCV disease. (B) Flow cytometric analysis of CD4 and CD8 expression by CD3$^+$ MNC isolated from liver samples showing normal histology (left), non-cirrhotic HCV chronic hepatitis (middle) and HCV cirrhosis (right). The numbers denote the percentages of cells in the four quadrants. (C) Two-colour immunofluorescence of normal liver sections (left), and liver sections from patients with non-cirrhotic (middle) and cirrhotic (right) HCV infection, showing expression of CD3 (red) and CD4 (green). Arrows indicate some of the cells staining positive for both markers (yellow). The number of CD3$^+$ cells that coexpress CD4 increase with severity of HCV-induced liver disease.
liver tissue sections confirmed a graded increase in CD4⁺ T cells in situ in HCV-infected liver, which correlated with disease progression. Hepatic CD4⁺ T cells were predominantly located in the periportal regions but were also observed scattered throughout the parenchyma (Fig. 2C).

3.4. Expansion of intrahepatic conventional αβ T cells and depletion of αβ CD56⁺ T cells in chronic HCV infection

The overall proportions of αβ T cells were increased in the livers of patients with non-cirrhotic HCV disease (median 71.6%, range 25.3–91.2%) compared to histologically normal livers (55.1%, 34.5–65.0%; \( P = 0.02 \); Fig. 3A) but γδ TCR⁺ lymphocytes were not significantly changed (Fig. 3B). When CD56⁺ and CD56⁻ T cells were analysed separately, the proportions of CD56⁻ αβ T cells were found to be significantly expanded in patients with non-cirrhotic HCV infection (64.5%, 23.0–91.2% vs. 39.4%, 24.4–49.4%; \( P = 0.02 \); Fig. 3C), while αβ CD56⁻ T cells were significantly depleted (3.5%, 0.9–20.3% vs. 12.8%, 8.6–16.3%; \( P = 0.02 \); Fig. 3E). Percentages of αβ CD56⁺ T cells were also depleted in patients with cirrhotic HCV disease (4.0%, 0.1–8.4%; \( P = 0.001 \); Fig. 3E). The frequencies of CD56⁻ and CD56⁺ T cells expressing γδ TCRs were similar in the three groups (Fig. 3D, F).

3.5. Depletion of intrahepatic Vα24 TCR⁺ T cells in chronic HCV infection

The proportions of T cells and CD56⁺ T cells expressing Vα24 TCR α-chains in histologically normal livers and in HCV-infected livers were compared using flow cytometry. Fig. 4A shows that compared to normal liver Vα24 TCR⁺ lymphocytes were reduced in patients with cirrhotic HCV disease (median 0.7%, range 0.3–2.7% vs. 2.3%, 1.2–9.6%; \( P = 0.02 \)). The reduction was mainly due to a decrease in Vα24⁺CD56⁻ T cells (0.3%, 0.05–0.9% compared with 1.4%, 0.5–6.2% in histologically normal livers; \( P = 0.01 \); Fig. 4C) but CD56⁻ T cells expressing Vα24 TCR α-chains were also slightly depleted (Fig. 4B).

3.6. Intrahepatic CD4⁺ T cells in chronic HCV infection are predominantly Th1 cells

Since CD4⁺ T cells were expanded in the livers of patients with chronic HCV infection, cytokine production by these cells was examined after stimulation ex vivo with PMA and ionomycin or anti-CD3 mAb. The proportions of CD3⁺CD4⁺ cells that stained positive for IFN-γ and IL-4 were determined by a combination of cell-surface and intracellular mAb staining and analysis by flow cytometry (Fig. 5).
5A, B). Intrahepatic CD4+ T cells from five normal livers produced IFN-γ (median 32.9%, 27.0–38.1%) while 4.9% (0.7–11.2%) produced IL-4. The proportions of IFN-γ-producing CD4+ T cells in three patients with cirrhotic HCV disease were significantly increased (median 54.3%, range 42.1–55.2%; P = 0.01; Fig. 5C) while the proportions of IL-4-producing CD4+ T cells were unchanged (Fig. 5C). Thus, the expanded CD4+ T cells in chronic HCV infection are likely to be Th1 cells. Ongoing production of IFN-γ was confirmed in non-cirrhotic HCV-infected liver tissue by immunohistochemistry (n = 4, Fig. 5D).

4. Discussion

The intrahepatic pool of lymphocytes is thought to play a key role in determining whether host immune responses to HCV infection result in viral clearance or immune pathology and chronic hepatitis [3]. Viral clearance is associated with potent HCV-specific CD8+ and CD4+ Th1 cell responses that occur in the early stages of infection [4,5]. Persistence of HCV is thought to be due to qualitative and/or quantitative inadequacies in these responses and also to the chronic activation of IFN-γ-producing cells that cause hepatocyte injury [3,4]. It is not known which cells are the primary orchestrators of immune-mediated damage. Hepatic NK cells, γδ T cells, CD4+ Th1 cells, CD8+ cytotoxic T cells (CTL) and CD56+ T cells can all produce IFN-γ [24] and CD8+ T cells, NK cells and NKT cells have been shown in various systems to mediate hepatocyte injury [10,16,25]. Expansions of αβ and γδ T cell subsets and depletions in the numbers of NK cells and CD56+ T cells have been reported to occur in the livers of chronically HCV-infected individuals [17,26].

The results of the present study provide evidence of significant MNC numbers in normal liver tissues and a small increase in the livers of patients with mild chronic HCV disease whilst a further increase (>2-fold) was seen in patients with end-stage HCV cirrhosis. Tissue manipulation required to prepare cell suspensions for flow cytometry may not have released all the cells, particularly from cirrhotic tissue. Our results confirm a previous report [26] that the proportions of CD56+ T cells are increased and CD56+ T cells are decreased in the livers of patients with chronic HCV infection compared with histologically normal donor livers. Our data suggest that the increase in CD56+ T cells is due to expansions of IFN-γ-producing CD4+ T cells bearing αβ TCRs, but not to γδ, CD8+, DN or DP T cells. In contrast, the proportions of αβ TCR+ T cells expressing CD56 are depleted in chronic HCV infection and this depletion includes a significant decrease in CD56+ T cells expressing the Vα24 TCR α-chain. Since fairly similar numbers of MNC were obtained per milligram of liver tissue from histologically normal and non-cirrhotic HCV patients, these proportional changes probably reflect the changes in absolute numbers. However, because cirrhotic livers yielded >2-fold higher numbers of MNC/mg than healthy liver, our observed proportional decreases of CD56+ and Vα24 TCR+ T cells may be due to increases in the numbers of other cell populations.

A significant proportion of human hepatic Vα24+ T cells express invariant TCR JαQ junctional sequences [9,24]. Invariant Vα24JαQ TCR chains pair with a limited number of β-chains forming a TCR that defines a subset of NKT cells which recognize glycolipid antigens presented by CD1d [12,13]. They express NKRs and activated phenotypes and notably can rapidly produce IFN-γ and IL-4 upon TCR stimulation [12,24]. Murine NKT cells expressing the homologous Vα14Jα281 TCR chain have potent anti-metastatic effects in the livers of mice [11,27] and appear to play a role in the inhibition of replication of HBV in HBV-transgenic mice [10]. These effects appear...
to be mediated by the secretion of IFN-γ which subsequently activate NK cells and CTLs. The decreased proportions of Vα24⁺ T cells in the livers of humans with chronic HCV infection found in the present study may result in insufficient activation of NK cells and CTLs required for elimination of the virus. It may also explain the susceptibility of patients with chronic HCV disease to hepatocellular carcinoma [1,2,26]. These hypotheses are supported by our observations that the proportions of intrahepatic NK cells and CD8⁺ T cells are also depleted in chronic HCV infection. Alternatively, the low numbers of intrahepatic NKT cells found in patients with chronic HCV disease may be due to activation-induced cell death, since Kakimi et al. [10] noted that in vivo stimulation of murine NKT cells in mice resulted in their subsequent disappearance.

In addition to Vα24⁺ T cells, other CD56⁺ T cells are clearly deficient in the livers of patients with chronic HCV infection. Vα24⁺ T cells generally account for <5% of hepatic CD56⁺ T cells and the reductions in Vα24⁺ T cells could not totally account for the reductions of CD56⁺ T cells. We have previously reported that non-Vα24⁺ hepatic T cells have similar properties to NKT cells, such as spontaneous cytotoxicity and dual IFN-γ and IL-4 production [24]. CD56⁺ T cells display properties of innate lymphocytes such as IL-12 responsiveness [28], invariant receptors for stimulatory ligands [6] and MHC-unrestricted cytotoxic function [24,28]. Failure to eliminate HCV may result from a deficiency of such innate lymphocytes at the early stages of infection. Innate lymphocytes can recognize conserved structures that signal viral infection [6,29,30] and may be required to rapidly resolve HCV infection before virus-specific T cells can differentiate into effector cells and proliferate to sufficient numbers to be effective.

NKT cells and CD56⁺ T cells are uniquely capable of being able to rapidly produce Th1 and Th2 cytokines upon stimulation [12,24,31], indicating a broader role for these cells in the activation and regulation of multiple arms of the immune response. Imbalances in Th1/Th2 cell activation are central to the failure of the immune system to eliminate viruses, bacteria and parasites. Th1/Th2 imbalances that appear to be associated with NKT cell deficiencies are also found in autoimmune disease [32–34]. Our data suggest that the cytokine balance in the livers of patients with chronic HCV infection may be shifted towards a Th1 response, with expansions of IFN-γ-producing CD4⁺ T cells. Whether these expanded Th1 cells are HCV-specific and whether they mediate hepatocyte damage is unknown.

While hepatic CD56⁺ T cells display properties of innate lymphocytes, they also express memory T cell phenotypes [9] and homing chemokine receptors [35] suggesting that they may be memory T cells. The upregulation of expression of CD56 and other NKRs by T cells, including virus-specific T cells can be induced by activation [8,20,21,36–38]. Thus, the deficiency of CD56⁺ T cells in the livers of patients with chronic HCV infection, seen in the present study, may alternatively represent a deficiency of memory T cells.

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