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# Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection☆

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***Background/Aims*: T lymphocyte-mediated immune reactions are closely involved in the pathogenesis of HCV-induced chronic liver disease. Regulatory T cells are able to suppress HCV-specific T lymphocyte proliferation and cytokine secretion during chronic HCV infection. We wished to address to what extent regulatory T cells exist in the livers of HCV+ individuals, and what the role of such cells might be in disease progression.**

***Methods*: We analysed the frequency and distribution of FOXP3+ cells, along with CD4, CD8 and CD20+ cells, in liver biopsies of 28 patients with chronic HCV and 14 patients with PBC, and correlated these data with histological parameters.**

***Results*: A striking number of FOXP3+ cells were present in the portal tract infiltrates of HCV-infected livers. FOXP3+ cells were largely CD4+ and there was a remarkably consistent ratio of total CD4+:FOXP3+ cells in liver across a wide range of disease states of around 2:1. This differed substantially from the ratio observed in PBC (10:1, $P = 0.001$).**

***Conclusions*: An unexpectedly high proportion of the cellular infiltrate in persistent HCV infection comprises FOXP3+ cells. The relative proportion of FOXP3+ cells remains similar in both mild and severe fibrosis. These cells are likely to play a critical role in intrahepatic immune regulation, although their overall role in disease progression remains to be determined.**



***Keywords*: Regulatory T cell; FOXP3; Hepatitis C virus; Disease progression; Liver**

## 1. Introduction

Persistence of hepatitis C virus (HCV) occurs in the majority of those who are initially infected with the virus. Viral persistence is associated with a wide range of clinical outcomes but may lead to the development of cirrhosis in up to 20–30% of individuals [1,2]. Progression of liver fibrosis towards cirrhosis is not clearly linked to virological factors, whereas factors such as age at infection, alcohol consumption, male gender and immunosuppressed states have been positively correlated [2–5]. T cell-mediated immune

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*Abbreviations:* HCV, hepatitis C virus; PBC, primary biliary cirrhosis; 3P3, forkhead box protein FOXP3; PBMC, peripheral blood mononuclear cell; CTLA, cytotoxic T lymphocyte antigen; IL, interleukin; TGF, transforming growth factor; IFN, interferon.

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reactions have also been proposed to play a role in progression [6–9]. Sustained HCV-specific T cell responses have been correlated with spontaneous resolution of acute HCV infection [10–12]. However in cases where virus persists, both CD4+ and CD8+ T cell responses appear to be attenuated markedly in blood [13–16], although they may persist in the liver, where they may contribute to disease pathogenesis [11,17,18]. Therefore, regulation of the T cell response in HCV patients has important implications not only in determining spontaneous clearance versus persistence, but also disease progression during persistent infection.

Regulatory T cells during HCV infection have been shown to inhibit both HCV-specific CD4+ and CD8+ T cell proliferation and cytokine secretion [19–23]. A problem with the study of regulatory T cells is the lack of a specific marker for cells with suppressor activity. In particular, many surface markers (e.g. CD25, CTLA-4 and glucocorticoid-induced TNF receptor) do not accurately represent all CD4+ T cells with regulatory activity, and overlap with activated T cells which do not necessarily possess regulatory activity. Human CD4+ CD25+ cells isolated from peripheral blood express FOXP3 have suppressive abilities [24–27] and are thought to include a population of thymically-derived regulatory T cells, such as those found in mice. Human CD4+ CD25+ cells can be activated to express FOXP3 (in conjunction with CD25), with CD4+ T cells derived from PBMC that have been induced, or already express FOXP3, having all the capabilities of regulatory T cells [28]. A recent study indicated that sustained high levels of FOXP3 expression as opposed to transient low level expression within activated CD4 or CD8 T cells are required to acquire the regulatory T cell phenotype [29]. Furthermore, antigen-specific regulatory T cells have been generated *in vitro* which are capable of suppression of unrelated T cell proliferation, but only in the presence of the cognate antigen [30].

Together, these data correlate well with the studies of regulatory T cells (based on CD4+ CD25+ expression) during persistent HCV infection, which indicate that regulatory T cells may play a significant role in the low frequency and dysfunction of HCV-specific T cell responses observed [17,31,32]. The regulatory T cell populations studied so far have cell-contact dependent mechanisms for inhibition of T cell proliferation [19,21] and are able to secrete TGF-β and IL-10, with TGF-β being able to inhibit the secretion of IFN-γ by HCV-specific T cells [20]. Additionally, HCV-specific CD8+ regulatory T cells have also been identified amongst lymphocytes derived from the livers of chronic HCV patients [23]. However, little is known of the frequency and distribution of regulatory T cells within liver tissue and how they correlate with inflammation and fibrosis. To evaluate this further, studies using a marker such as FOXP3 that identifies cells with regulatory capacity are required. Recently, a panel of FOXP3 monoclonal antibodies were made which provide a valuable specific research tool for examination of regulatory T cells *in situ* [33].

We hypothesized that within HCV-infected livers, the frequency of FOXP3+ cells might be linked to clinical and histological parameters of disease. Potentially, a decrease in relative numbers would allow dysregulation of T cell-mediated immunity and predispose to inflammation and fibrosis. Alternatively, an increase in frequency may increase TGF-β secretion within the liver, which would assist in the development of fibrosis by activated hepatic stellate cells [34–36]. Defining these intrahepatic phenomena is crucial to understanding the immunopathogenesis of HCV infection.

## 2. Materials and methods

### 2.1. Liver histology

Formalin-fixed, paraffin-embedded liver biopsy specimens from 28 HCV and 14 primary biliary cirrhosis (PBC) patients were obtained from the archive of the Department of Pathology at the John Radcliffe Hospital, Oxford, UK. These were selected to represent a range of disease stages – from very mild to cirrhosis. In addition, tonsil tissues were used for both positive and negative controls. Ethical approval for the study was obtained from the local Ethical Review Panel (COREC) and the protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### 2.2. Immunohistochemistry and double immunoenzymatic labelling

First, 5-μm-thick paraffin-embedded tissue sections on electrically charged slides (Snowcoat X-tra, Surgipath) were dewaxed in Citroclear (HD Supplies, Aylesbury, UK), rehydrated,and then submitted to antigen retrieval by heating in 50 mM Tris and 2 mM EDTA, pH 9.0, in a microwavable pressure cooker at full pressure for 10 min. After cooling, the slides were placed in phosphate-buffered saline (PBS) for 5 min. Endogenous peroxidase was blocked by means of a commercial peroxidase-blocking reagent (DakoCytomation, Glostrup, Denmark) for 5 min in a humidified chamber at room temperature. Slides were rinsed briefly in PBS/0.02% Tween 20 (PBS-T) then incubated for 30 min with mouse anti-human FOXP3 antibody, clone 236A/E7 [33]. The slides were washed for 5 min in PBS-T and immunodetection was performed with a polymer HRP-labelled anti-mouse secondary antibody for 30 min, followed by peroxidase-labelled streptavidin for 30 min and DAB chromogen as substrate for 10 min (EnVision system, DakoCytomation). Single stain sections were counterstained with hematoxylin Gill No. 3 (Sigma). Double immunoenzymatic labelling with CD4, CD8 or CD20 was carried out as described above with the second reaction detected by means of a Vector SG blue horseradish peroxidase substrate kit (VectorLaboratories, Peterborough, UK). The sections were washed in tap water and mounted in aquamount (Merck, Poole, UK). All sections were stained in parallel with an unrelated primary antibody (MR12) to provide a negative control for each reaction.