The Intrahepatic T Cell Compartment Does Not Normalize Years After Therapy-Induced Hepatitis C Virus Eradication

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Little is known about the immune status in liver and blood of patients with chronic hepatitis C virus (HCV) infection long after therapy-induced viral clearance. In this study, we demonstrate that, 4 years after clearance, regulation of HCV-specific immunity in blood by regulatory T cells (Tregs) and the immunosuppressive cytokines interleukin 10 and transforming growth factor β is still ongoing. Importantly, analysis of liver specimens collected 4 years after HCV clearance shows that intrahepatic Tregs are still present in all patients, suggesting that liver T cells remain regulated. Identifying mechanisms that regulate HCV-specific memory T-cell responses after clearance is highly relevant for the development of protective vaccines, especially in patients at high risk of reinfection.

Keywords: intrahepatic; gene expression; Treg; IL-10; TGF-β.

Virus-specific T-cell responses are key players in controlling infection with hepatitis C virus (HCV). During chronic HCV infections, these T cells are present at low frequencies and are dysfunctional [1, 2]. Critical factors contributing to the weak T-cell responses include active suppressive regulation by FoxP3+CD25+CD4+ regulatory T cells (Tregs) and inhibitory cytokines, such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β), as well as T-cell exhaustion due to continuously high viral antigen levels [1, 3]. In a previous study, we showed that, 4 weeks after therapy-induced viral clearance, negative regulatory mechanisms involving IL-10, TGF-β, and Tregs are still functional in controlling HCV-specific T-cell responses in peripheral blood [4].

Although HCV replication takes place in the liver, little information is available in humans on intrahepatic T-cell immunity after therapy-induced viral clearance. We showed that, prior to therapy but also 4 weeks after the end of interferon-based antiviral treatment, Treg are present in the liver of patients with chronic HCV infection [5]. This is in contrast to livers never exposed to HCV, which contain virtually no Treg [6], and this suggests that intrahepatic virus-specific T-cell responses remain regulated by Treg shortly after viral clearance. Retention of Treg has also been demonstrated in mouse skin after clearance of the stimulus following primary antigen exposure [7]. These skin Treg were shown to possess memory cell characteristics since antigen re-expression led to more efficient suppression of T-cell responses by these memory Treg and less severe skin disease as compared to the situation following primary antigen exposure [7, 8].

Identification of mechanisms responsible for the failure of the HCV-specific T-cell response to create long-term protective immunity are important especially for the development of protective vaccines and for patients at high risk of reinfection. At present, it is unknown whether intrahepatic Treg are retained in the liver after therapy-induced viral clearance and whether these cells have similarities to memory Treg observed in mouse skin and in human. In this study, we evaluate patients with a sustained viral response following interferon-based therapy 4 years earlier by sampling the liver. In addition, HCV-specific T-cell responses and mechanisms for regulation in peripheral blood are studied.

MATERIALS AND METHODS

Patients

In the present study, patients with chronic HCV infection were included from our previous cohort of 21 treatment-naive patients who were treated with pegylated interferon alfa 2a (PegInteron; MSD, Houten, the Netherlands) and ribavirin (Rebetol; MSD) and who were evaluated longitudinally [5]. Five patients, all with a sustained viral response, volunteered to participate in this study and donated blood specimens; of these, 4 also underwent additional sampling of the liver by fine-needle aspiration.
The institutional ethical board of the Erasmus MC approved the clinical protocol for this follow-up study, and written informed consent was obtained from all individuals. Patients’ characteristics are listed in Supplementary Table 1, and patients’ study numbers coincide with those in our previous article, to allow full transparency of the clinical and immunological data from the individual data at all time points [5].

Aspiration of Liver Cells and Collection of Peripheral Blood
Intrahepatic leukocytes were obtained via fine-needle aspiration as described elsewhere [5]. For microarray analyses, blood was collected in Tempus Blood RNA tubes (ABI, Foster City, California). Samples were collected 4 years after ending of therapy and compared with historical data at baseline and at weeks 4 and 24 after ending HCV therapy. Flow cytometry to analyze cell surface molecule expression was performed on fresh whole-blood specimens or liver aspirate biopsy specimens, using methods similar to those described previously [4]. Fresh peripheral blood mononuclear cells (PBMCs) were used to quantify HCV-specific T-cell proliferation, by [3H]-thymidine incorporation on day 6, interferon γ (IFN-γ) production, by enzyme-linked immunosorbent assay on day 3. Cultures were stimulated with HCV overlapping peptide pools, supplemented with antibodies against IL-10 receptor or TGF-β or following depletion of Tregs from among PBMCs [3–5]. Polyclonal stimulations, using soluble anti-CD3 antibodies or cytomegalovirus (CMV) lysate, were used as positive controls.

Gene Expression Analysis
Methods of sample preparation, microarray analysis, and processing of array data are described elsewhere [9] and in the legend to Supplementary Figure 2. Microarray data were deposited in the Gene Expression Omnibus (accession number GSE64603).

RESULTS

Negative Regulation of HCV-Specific T Cells Is Still Present After Long-Term Viral Clearance
Besides Tregs, IL-10 and TGF-β are well-described negative regulators of virus-specific T-cell proliferation and IFN-γ production in various viral infections [6, 10], as well as during therapy of patients with chronic HCV infection [10]. We evaluated whether these mechanisms continue to regulate HCV-specific T-cell responses in PBMCs from 5 patients 4 years after therapy-induced HCV eradication. Patient characteristics are described in Supplementary Table 1. Although HCV RNA was undetectable in serum specimens from each patient, HCV-specific T-cell proliferation was observed after stimulating PBMCs with an overlapping HCV peptide pool (Figure 1). Importantly, depletion of Tregs or neutralization of TGF-β in these cultures resulted in enhanced virus-specific T-cell proliferation (Figure 1). We observed enhanced T-cell proliferation upon Treg depletion in 3 of 5 cases and stronger T-cell proliferation upon TGF-β neutralization in 2 of 5 patients. PBMCs stimulated with CMV lysate were used as positive controls, the analysis of which demonstrated that Treg depletion resulted in increased CMV-specific T-cell responses (data not shown). In contrast to T-cell proliferation against HCV, no virus-specific IFN-γ production was detected in culture supernatant of PBMCs stimulated with peptides after 4 years of follow-up (Figure 1). However, when the IL-10 receptor was blocked, IFN-γ levels were enhanced in 2 of 5 patients, and when TGF-β was blocked, IFN-γ levels were enhanced in 2 other patients. Together, we show here that HCV-specific memory T cells persist in peripheral blood 4 years after therapy-induced viral eradication, are functionally responsive to HCV antigens, and continue to be regulated by various inhibitory mechanisms.

To determine the status of the T-cell compartment of patients 4 years after viral clearance at the transcript level, we evaluated the expression of 244 probe sets (corresponding to 121 genes) that are important for T-cell function and immunoregulation. The list of genes is presented in Supplementary Table 2. Twenty-one genes were still modulated 4 years after therapy-induced viral clearance in blood (fold change, 1.5; P < .05; Supplementary Table 3). The genes with the highest fold increase were TGFBR1 and TGFBR1, which may indicate a stronger involvement of TGF-β-mediated regulation 4 years after viral clearance, compared with baseline.

Retention of Tregs in the Liver 4 Years After Therapy-Induced Clearance of HCV
By repeated sampling of the liver, we previously demonstrated retention of intrahepatic Tregs in patients 4 weeks after end of treatment [5]. Furthermore, we also previously showed that virtually no Tregs were observed in healthy livers [6]. To evaluate whether intrahepatic Tregs remain in the liver long after viral clearance, liver specimens from 4 of the 5 patients described above were obtained 4 years after sustained viral response. As shown in Figure 2A, Tregs were defined as CD45+CD3+CD4+ lymphocytes with high expression of CD25 and FoxP3. Four years after sustained viral response, we observed Tregs in the liver of all 4 patients, with frequencies varying from 3.0% to 10.7% of the total CD4+ T-cell population. Comparison of these Treg frequencies with those detected follow-up weeks 4 and 24 showed that their numbers were retained at relatively high levels. Phenotypic characterization demonstrated that, on average, 23% of Tregs displayed an effector memory phenotype (ie, CD45RO+CD62L−), which was in line with their phenotype 4 weeks after the end of treatment (Figure 2B). Frequencies and phenotypes of Tregs in peripheral blood were comparable to those for healthy individuals or at various time points during therapy, as presented in our previous study [4]. Overall, these results indicate that, even 4 years after HCV eradication, intrahepatic Tregs with a memory
phenotype remain present in the liver for an extended period despite the absence of detectable antigen.

**DISCUSSION**

This study shows that, years after therapy-induced HCV clearance, Tregs remain present in the liver, despite the absence of detectable serum HCV RNA. Besides regulation via Tregs in the liver, we also observed in peripheral blood that, after virus eradication, HCV-specific T-cell responses remain suppressed by Tregs, TGF-β, or IL-10 4 years after elimination of HCV RNA. In addition, a role for these mechanisms was supported by their gene expression profile, which demonstrated abundant transcripts of factors in the TGF-β pathway after therapy-induced HCV clearance.

During the chronic phase of HCV infection, Tregs are present at relatively high numbers in the liver, which is in contrast to healthy livers, which harbor virtually no Tregs (only 2% of the CD4+ T-cell population, as revealed in our previous study [6]). It is generally accepted that, during the chronic phase, these intrahepatic Tregs control HCV-specific T-cell responses and likely prevent immunopathology [6]. Interestingly, our data demonstrate that Tregs are still present in the liver 4 years after completion of successful interferon-based therapy. An interesting explanation for the presence of Tregs in the liver after viral clearance could be that these regulatory mechanisms are maintained to limit fibrogenesis, since Tregs and IL-10 have been shown to limit pathology in HCV-infected patients [11]. Interestingly, at the transcript level, we demonstrated that, among other regulatory genes, expression of genes involved in TGF-β regulation (TGFBR1 and TGFBI) is upregulated 4 years after viral clearance. Besides TGF-β, we also observed that other genes involved in the TGF-β pathway were differentially expressed 4 years after virus eradication, compared with before.
therapy (Supplementary Figure 1). However, these results need to be interpreted with caution, since gene expression profiling was performed on whole-blood specimens. Differential expression of the TGF-β pathway after long-term virus eradication does support our data on regulation and can be especially important for future studies on fibrogenesis, since a role for TGF-β in the promotion, as well as the inhibition, of fibrogenesis via hepatic stellate cells has been extensively described [12]. In this article, we clearly demonstrate that, besides TGF-β, factors such as Tregs and IL-10 play a role in regulating HCV-specific T-cell responses after viral clearance. A better understanding of the role for Tregs, TGF-β, and IL-10 in the immunopathogenesis of fibrosis can lead to development of therapeutic targets to delay this process.

Phenotypic characterization of intrahepatic Tregs 4 years after cessation of therapy showed predominantly a CD45RO⁺CD62L high central memory phenotype, indicating a less active state, compared with the chronic phase of HCV infection. This phenotype
is in line with memory Tregs observed in mouse and human skin [7,13]. Because of low numbers of cells that can be isolated from a fine-needle aspiration biopsy specimen, we were unable to further investigate the putative memory function of Tregs in our patients and to demonstrate whether the proliferative capacity and cytokine profile resemble that of mouse memory Tregs [13].

Another explanation for the retention of Tregs long after viral eradication is the detection of low amounts of HCV RNA in the liver and PBMCs of patients after viral clearance. Interestingly, these low amounts of HCV RNA are able to stimulate cellular immune responses [14]. It is tempting to speculate that the active regulatory mechanisms observed in peripheral blood and the presence of intrahepatic Treg long after viral clearance are present to control or attenuate sterilizing HCV-specific T-cell responses targeting residual HCV RNA. This way, local and low-level viral persistence can be established that may benefit the host by maintaining a functional HCV-specific memory T-cell pool. On the hand, these regulatory mechanisms may instead dampen HCV-specific recall responses and prevent protection against reinfection. One study detected HCV-specific T cells after successful interferon-free therapy in a chimpanzee with chronic HCV infection. These CD8+ T cells had a narrow antigenic specificity, were weak, and were unable to prevent persistence upon secondary infection [15].

Vaccination after cure to broaden otherwise narrowly focused CD8+ T-cell memory may provide protection from reinfection, especially in patients who have been successfully treated but remain at risk for virus exposure, such as men who have sex with men or injection drug users. The presence of negative regulatory mechanisms in blood and liver after viral clearance needs to be taken into account when designing such a protective vaccine.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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