Towards optimal treatment for chronic hepatitis C infection

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Monocyte derived dendritic cells from chronic HCV patients are not infected but show an immature phenotype and aberrant cytokine profile

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dendritic cells – hepatitis C – monocytes – therapeutic vaccination

Abstract

Background: Chronic hepatitis C virus (HCV) infection is characterized by an insufficient immune response, possibly owing to impaired function of antigen-presenting cells such as myeloid dendritic cells (DCs). Therapeutic vaccination with in vitro generated DCs may enhance the immune response. Subsets of DCs can originate from monocytes, but the presence of HCV in monocytes that develop into DCs in vitro may impair DC function. Therefore, we studied the presence of HCV RNA in monocytes and monocyte-derived DCs from chronic HCV patients.

Methods: Monocytes were cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) for 6 days, and then with GM-CSF, IL-4, tumour necrosis factor-α (TNF-α), prostaglandin E2, IL-1β and IL-6 for 2 days to generate mature DCs. HCV RNA was assessed by polymerase chain reaction. Surface molecules were assessed by flow cytometry. Cytokine production was assessed by cytokine bead array.

Results: HCV RNA was present in monocytes in 11 of 13 patients, but undetectable in mature DCs in 13 of 13 patients. The morphology of patient DCs was comparable with DCs from healthy controls, but the percentage of cells expressing surface molecules CD83 (P = 0.001), CD86 (P = 0.023) and human leucocyte antigen-DR (P = 0.028) was lower in HCV patients. Compared with control DCs, patient DCs produced enhanced levels of IL-10 (P = 0.0079) and IL-8 (P = 0.0079), and lower levels of TNF-α (P = 0.032), IL-6 (P = NS) and IL-1β (P = 0.0079). Patient and control DCs did not produce IL-12.

Conclusions: Monocyte-derived DCs from chronic HCV patients are not infected but show an immature phenotype and aberrant cytokine profile.

After acute hepatitis C virus (HCV) infection, approximately 75% of the patients do not clear the virus and develop chronic infection (1). The current antiviral therapy based on administration of pegylated interferon (IFN)-α and ribavirin for 24–48 weeks leads to a sustained virologic response (SVR) in approximately 40% of patients infected with HCV genotype 1. IFN-α and ribavirin therapy is expensive and causes significant side effects in most patients. Up to 20% of patients stop therapy prematurely because of side effects. Thus, there is a clinical need for more effective therapies. Determination of viral and host immunological mechanisms responsible for the persistence of HCV infection may facilitate development of new treatment strategies.

Spontaneous clearance of HCV infection is characterized by a strong and broad HCV-specific CD8+ T cell and CD4+ T cell response that persists for years after resolution of the acute infection (2–4). In chronic HCV infection, the HCV-specific CD8+ T cell and CD4+ T cell response is generally weak and narrow (2, 3). Interestingly, most patients who eventually develop chronic infection also exhibit strong HCV-specific CD8+ T cell and CD4+ T cell responses during the
acute phase of infection. However, the CD4⁺ T cell responses are not sustained, and these patients become chronically infected (5, 6). The reasons for this apparent failure of CD4⁺ T cell help are unknown. HCV also interferes with intracellular host defence mechanisms (7). Studies on the treatment of acute HCV have shown response rates of 90–100% with IFN-α monotherapy, regardless of the HCV genotype (8). The fact that HCV is easier to treat when the infection is recent (<6 months in duration) strongly suggests HCV-mediated changes in host immunity. Both host and virus factors are likely to play a role in the development and persistence of chronic HCV infection.

Recently, impairments in the function of dendritic cells (DCs) have been described in chronic HCV infection (9–12). DCs are professional antigen-presenting cells (APCs) that have the unique capacity to activate and polarize naïve Th cells (13, 14). Two major subpopulations of DCs are the plasmacytoid and myeloid DCs. Myeloid DCs are classical APCs that produce IL-12 and prime for IFN-γ-producing Th1 responses, whereas plasmacytoid DCs produce IFN-α in response to virus infection.

A promising method to enhance the cellular immune response is therapeutic vaccination with DCs. DC-based vaccines against various cancers, HCV and HIV have shown promising results (15–20). DCs can be generated from peripheral blood monocytes ex vivo, loaded with relevant peptides, DNA or mRNA, and subsequently readministered to the same patient where they will prime naïve T cells. However, the presence of HCV has been demonstrated in all major peripheral blood mononuclear cell (PBMC) subpopulations of chronic hepatitis C patients: B cells (21–27), CD4⁺ T cells (21, 23, 24), CD8⁺ T cells (21–24, 26), monocytes (21–23), and DCs (10, 25–27). Furthermore, monocyte-derived DCs can be infected in vitro (28). Thus, if ex vivo generated autologous DCs are to be used as a therapeutic vaccine during antiviral treatment, they need to be free of HCV RNA; otherwise, the patient might become reinfected by the vaccine, and patient-derived DCs used for therapeutic vaccination must be functionally normal.

Given that HCV can infect monocytes and DCs, and that myeloid DCs originate from monocytes (29), we evaluated (1) the presence and quantity of HCV RNA in peripheral blood monocytes and in monocyte-derived DCs from patients infected with HCV, and (2) the morphology and function of these DCs.

**Materials and methods**

**Patients**

We studied 13 patients with chronic HCV genotype 1 infection; the outline of the study is depicted in Table 1. The study protocol was approved by the ethics committee of our hospital, and all patients gave written informed consent. All patients were negative for hepatitis B surface antigen (HBsAg) and anti-HIV. Three patients were treatment naïve, and 10 patients were non-responders after IFN/ribavirin antiviral therapy. In all previously treated patients, treatment had been discontinued at least 6 months before sample collection. The clinical characteristics of the patients

**Table 1. Study diagram**

<table>
<thead>
<tr>
<th>Patient series</th>
<th>B (n = 5)*</th>
<th>Healthy controls (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes obtained via HCV RNA detection</td>
<td>Venipuncture</td>
<td>Leucapheresis</td>
</tr>
<tr>
<td>Generation of DCs</td>
<td>Qualitative and quantitative PCR on CD14CD45 sorted monocytes &amp; lymphocytes</td>
<td>Qualitative and quantitative PCR on CD14CD45 sorted monocytes &amp; lymphocytes</td>
</tr>
<tr>
<td>Culture with GM-CSF &amp; IL-4 followed by culture with GM-CSF, IL-4, TNF-α, PGE-2, IL-1β, IL-6</td>
<td>Culture with GM-CSF &amp; IL-4 followed by culture with GM-CSF, IL-4, TNF-α, PGE-2, IL-1β, IL-6</td>
<td>Culture with GM-CSF &amp; IL-4 followed by culture with GM-CSF, IL-4, TNF-α, PGE-2, IL-1β, IL-6</td>
</tr>
<tr>
<td>HCV RNA detection in iDCs and DCs</td>
<td>Qualitative PCR</td>
<td>Qualitative PCR</td>
</tr>
<tr>
<td>Maturation status surface markers</td>
<td>FACS</td>
<td>FACS</td>
</tr>
<tr>
<td>Cytokine secretion</td>
<td>ND</td>
<td>CBA</td>
</tr>
</tbody>
</table>

*Four of five patients in these series were also included in A.*

CBA, cytometric bead array; DCs, dendritic cells; FACS, fluorescence-activated cell sorting; GM-CSF, granulocyte macrophage colony-stimulating factor; HCV, hepatitis C virus; iDCs, immature dendritic cells; IL, interleukin; NA, not applicable; ND, not done; PCR, polymerase chain reaction; PGE2, prostaglandin E2; TNF, tumour necrosis factor.
are summarized in Table 2. All healthy controls were negative for anti-HCV, HBsAg and anti-HIV.

Specimen collection

For generation of DCs, we collected heparinized whole blood via venipuncture or PBMCs via leukapheresis. Monocytes were isolated from heparinized whole blood or from the leukapheresis product, diluted with an equal volume of phosphate-buffered saline (PBS) (NPBI International BV, Emmer Compascuum, the Netherlands), by sedimentation over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by sedimentation over Percoll (Amersham Pharmacia Biotech). The monocytes were collected and washed three times with PBS (NPBI International BV). Also, additional ethylene diamine tetraacetic acid and heparinized blood was drawn for determination of plasma HCV RNA, routine chemistry and haematology and additional isolation of PBMCs for cell sorting.

Cell sorting for detection and quantification of HCV RNA

PBMCs were isolated from heparinized whole blood, diluted with an equal volume of PBS (NPBI International BV), by sedimentation over Ficoll-Paque Plus (Amersham Pharmacia Biotech). Monocytes and lymphocytes were isolated from the PBMCs using fluorescence-activated cell sorting (FACS). Briefly, PBMCs were incubated with monoclonal antibodies anti-CD14-PE and anti-CD45-PE-Cy5 (IQ Products, Groningen, the Netherlands). CD14<sup>+</sup> CD45<sup>+</sup> cells (monocytes) and CD14<sup>-</sup> CD45<sup>+</sup> cells (lymphocytes) were then sorted to >98% purity using a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA) and collected directly in lysis buffer L6 (30) after sorting. The purity of the sorted cells was analysed using a FACScalibur flow cytometer (BD Biosciences) and CELLQUEST software (BD Biosciences).

Detection and quantification of HCV RNA in monocytes, lymphocytes and DCs

Qualitative polymerase chain reaction (PCR) for detection of HCV RNA in monocytes, lymphocytes and DCs was performed by modification of the Roche COBAS Amplicor HCV Test v2.0 (Roche Diagnostics, Branchburg, NJ, USA; lower limit of detection 50 IU/ml) as described previously (31). PCR for quantification of HCV RNA in monocytes and lymphocytes was performed by modification of the quantitative Roche COBAS Amplicor HCV Monitor Test, v2.0 (Roche Diagnostics; linear dynamic range 6 x 10<sup>2</sup>–5 x 10<sup>5</sup> HCV RNA IU/ml) as described previously (31). Owing
to elution of extracted RNA in a lower than usual volume, the lower limit of quantification of the modified quantitative PCR was $1.2 \times 10^5$ HCV RNA IU/ml (five times lower than the normal lower limit of quantification of $6 \times 10^5$ HCV RNA IU/ml).

### Quantification of HCV RNA in plasma

Plasma HCV RNA was assessed by quantitative bDNA (VERSANT HCV 3.0 assay; Bayer Diagnostics, Berkeley, CA, USA; linear dynamic range $615-7.7 \times 10^6$ IU/ml) (32).

### Generation of monocyte-derived DCs

Monocyte-derived DCs were generated according to previously described methods with minor modifications (33). Briefly, the monocytes were incubated for 6 days in ex vivo 15 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 1000 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF; Berlex Laboratories Inc., Richmond, CA, USA) and 1000 U/ml interleukin 4 (IL-4; R&D Systems, Minneapolis, MN, USA).

To generate mature DCs, the immature DCs were stimulated on day 6 in ex vivo 15 medium supplemented with a cytokine cocktail containing TNF-α (10 ng/ml; R&D Systems) prostaglandin E2 (PGE2; 1 μg/ml; R&D Systems), IL-1β (10 ng/ml; R&D Systems), IL-6 (150 ng/ml; R&D Systems) and GM-CSF (800 U/ml; R&D Systems) and IL-4 (500 U/ml; R&D Systems). After 24 or 48 h, the cells were harvested for further analysis. Immature DCs and mature DCs were collected for detection of HCV RNA on days 6 and 8 respectively. In two patients, cells were collected for detection of HCV RNA every day until day 8.

### Surface markers of DCs

Surface markers of mature DCs were examined by flow cytometry using a FACSCALIBUR and CELLQUEST software (BD Biosciences) with a panel of monoclonal antibodies: anti-CD14-FITC (BD Biosciences), anti-CD83-PE (BD Biosciences), anti-CD209-PE (DC-SIGN; BD Biosciences), anti-CD80-FITC (BD Biosciences), anti-CD86-FITC (BD Biosciences), anti-HLA-DR-PE (BD Biosciences) and anti-CCR7-PE (Mckinley, Minneapolis, MN, USA). After antibody binding and two washings with PBS, the cells were fixed with 4% paraformaldehyde. The cells were gated according to their forward and side scatter profile, and surface marker expression of the large and granular cell population was analysed.

### Cytokine production by DCs

The production of IL-12p70, IL-8 and IL-10 was assessed in the culture supernatant of mature DCs by cytometric bead array (BD Biosciences) in a subset of five randomly selected patients and five healthy controls. IL-6, TNF-α and IL-1β levels were also assessed, but these cytokines were already present in the maturation mix.

### Genotyping

HCV genotypes were determined using the TruGene HCV genotyping assay (Bayer Diagnostics).

### Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM version 4.0b for Macintosh (GraphPad Software, San Diego, CA, USA). The association between plasma HCV RNA, on the one hand, and HCV RNA in monocytes or lymphocytes, on the other, was determined using linear regression. The Mann–Whitney two-tailed t-test was used for comparison of surface markers and cytokine profiles of mature DCs. $P < 0.05$ was considered to be significant.

### Table 3. Summary of bDNA of HCV RNA in plasma, and PCR analysis of HCV RNA in monocytes, lymphocytes and DCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detection of HCV RNA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td>1.1</td>
<td>+</td>
</tr>
<tr>
<td>1.2</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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<td>3</td>
<td>+</td>
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<td>4</td>
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<td>5.1</td>
<td>+</td>
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<td>5.2</td>
<td>+</td>
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<td>6</td>
<td>+</td>
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<td>7</td>
<td>+</td>
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<td>8</td>
<td>+</td>
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<td>9</td>
<td>+</td>
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<tr>
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<td>+</td>
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<tr>
<td>10.2</td>
<td>+</td>
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<tr>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>12.1</td>
<td>+</td>
</tr>
<tr>
<td>12.2</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
</tr>
</tbody>
</table>

*HCV RNA < 615 IU/ml but positive by PCR (HCV RNA > 50 IU/ml). Patients 1, 5, 10 and 12 were studied twice.

DCs, dendritic cells; HCV, hepatitis C virus; PCR, polymerase chain reaction.
Chapter 8

Results

HCV RNA is present in monocytes and lymphocytes of HCV patients

We first examined the presence and quantity of HCV RNA in monocytes and lymphocytes of HCV-infected patients. CD14^+ CD45^+ monocytes and CD14^- CD45^+ lymphocytes were sorted to > 98% purity and analysed for HCV RNA by PCR. As depicted in Table 3, HCV RNA was detectable in CD14^+ CD45^+ monocytes of 11 of 13 patients (mean 6.9 x 10^3 HCV RNA IU/10^6 cells, range 3.2 x 10^3–6.1 x 10^4), and in CD14^- CD45^+ lymphocytes of 12 of 13 patients (mean 1.9 x 10^4 HCV RNA IU/10^6 cells, range 1.1 x 10^3–2.1 x 10^4). The mean plasma HCV RNA load of all 13 patients was 5.9 x 10^5 IU/ml (range 1.8 x 10^2–3.3 x 10^6). Linear regression analysis (Fig. 1a) revealed a significant association between plasma HCV RNA and HCV RNA in monocytes (P = 0.0163) and lymphocytes (P = 0.0074). The HCV RNA plasma load was a factor 20–1300 higher than the amount of HCV RNA in cells. As depicted in Fig. 1b, HCV RNA was consistently higher in lymphocytes compared with monocytes. These data indicate that a fraction of both the peripheral blood monocytes and lymphocytes of chronic hepatitis C patients are infected with HCV.

No evidence of HCV RNA in monocyte-derived DCs of HCV patients

We next examined the presence of HCV RNA in monocyte-derived DCs of chronic hepatitis C patients. In 2 of 13 patients, decreasing amounts of HCV RNA were detectable in immature DCs for up to 6 days of culture. In all other patients, HCV RNA was undetectable after 1 day of culture. We did not detect HCV RNA in mature DCs in any of the 13 patients, despite the high sensitivity of the RT-PCR (50 HCV RNA IU/ml ~100 HCV RNA IU/10^6 cells). Taken together, the above data indicate that uninfected DCs can be generated ex vivo from HCV-infected monocytes.

Decreased expression of surface molecules CD83, CD86 and HLA-DR on monocyte-derived DCs of HCV patients

We next examined the appearance and surface markers of monocyte-derived DCs of chronic hepatitis C patients and healthy controls. The morphology of the mature DCs showed large refractile cells of irregular shape with long cytoplasmic processes, and this was comparable to mature DCs from healthy controls (data not shown). Mature DCs from HCV-infected patients expressed the surface markers CD80, CD-209 and the lymph node homing receptor CCR7, in amounts that are characteristic for normal DCs (Table 4, Fig. 2). However, compared with control DCs, significantly less cells from HCV-infected patients expressed the surface molecules CD83 (P = 0.001), CD86 (P = 0.023) and HLA-DR (P = 0.028). These results indicate that monocyte-derived DCs of chronic hepatitis C patients do not have a normal pattern of expression costimulatory molecules and DC-specific cell surface markers, and thus an immature phenotype.

Aberrant cytokine production by monocyte-derived DCs of HCV patients

Priming of T cells depends on DC functions such as secretion of cytokines. We examined the cytokine...
production of DCs from five randomly selected patients and five healthy controls. We harvested the culture supernatant of mature DCs and assessed the levels of IL-12p70, TNF-α, IL-10, IL-6, IL-1β and IL-8 (Fig. 3). Compared with healthy controls, the patient-derived DCs produced IL-10 and very high levels of IL-8. IL-12p70 was detected in minute amounts in only one of five patients, and in two of five healthy controls. The amounts of IL-10 produced varied considerably between DCs from different patients (range 58–2570 pg/ml). In contrast to control DCs, patient DCs produced high levels of IL-8. Although added to the maturation mix in equal amounts, we observed significant differences in the levels of TNF-α, IL-6 and IL-1β between patient DCs and control DCs (Fig. 3). Compared with control DCs, patient DCs produced less TNF-α, less IL-6 and less IL-1β. These results suggest that cytokine production by monocyte-derived DCs of chronic hepatitis C patients is abnormal.

**Discussion**

In the present study, we have demonstrated that monocytes from chronic hepatitis C patients contain HCV RNA, but that DCs generated ex vivo from these monocytes are free of HCV RNA. The DCs express lower amounts of costimulatory molecules, and the pattern of cytokine secretion is not normal. Our findings, which are suggestive of an immature phenotype and an impaired capacity of monocyte-derived DCs from chronic HCV patients to prime T_{H1} responses, the principal aim of therapeutic vaccination in chronic viral infections, question the feasibility of
therapeutic vaccination approaches with autologous monocyte-derived DCs in chronic HCV infection.

Even though the monocytes from 11 of 13 chronic hepatitis C patients contained HCV RNA, the monocyte-derived DCs were free from HCV RNA in all cases. The rapid disappearance of HCV RNA after 1 day of culturing in all but two patients suggests a direct effect of HCV on infected cells, resulting in the death of infected monocytes during stimulation, or that the ex vivo conditions used in our study were suboptimal for sustained infection.

The HCV RNA levels were lower in monocytes compared with lymphocytes. This may be related to HCV cell tropism; alternatively, the longer life span of lymphocytes may increase their chance of infection.

The majority (10/13) of patients in this study were non-responders to IFN treatment. Therefore, the presence of HCV RNA in monocytes (9/10) and lymphocytes (10/10) in most of these patients may be a cause and/or a consequence of IFN non-response.

Lower percentages of cells expressing CD83 and CD86 have been described previously in chronic HCV patients (9, 11). Compared with control DCs, the low expression of CD83, CD86 and HLA-DR and the aberrant cytokine pattern of patient DCs – with a higher secretion of IL-10 and IL-8 but a lower secretion of IL-6, IL-1β, TNF-α and no secretion of IL-12 – suggest an HCV-mediated countermechanism against the innate immune response with direct consequences for the cellular immune response (7).

The secretion of IL-10 but not IL-12p70 by DCs derived from chronic HCV patients suggests that these DCs would induce T_{H12} responses or regulatory T-cell responses (34). This may have been caused by the presence of PGE2 in the maturation mix (35, 36). Maturation mixes containing PGE2 result in phenotypically normal but functionally compromised DCs (35, 36). Low IL-12p70 and high IL-10 production by circulating DCs from chronic HCV patients has been described previously (37). These results suggest an HCV-mediated effect on DC function, possibly by secretion of IL-10. Both NS3 (38) and NS4 (39) stimulate monocytes to produce IL-10. The aim of therapeutic vaccination in chronic HCV infection is to enhance T_{H11} responses, but not induction of T_{H2} responses or regulatory T-cell responses. Thus, our results imply that these DCs would be of limited benefit.

Previous studies have shown that serum IL-8 is elevated in chronic HCV infection (40); this is mediated by NS5A (41) and the main source of IL-8 might be infected monocytes. Here, we show that monocyte-derived DCs from chronic HCV patients secrete high amounts of IL-8 compared with control DCs. Whether this enhanced production is mediated by NS5A remains to be established. Both high (42) and low (35) levels of IL-8 have been observed in DCs from healthy controls in other studies but with different maturation cocktails. The low levels of IL-8 secreted by DCs from healthy controls vs high levels of IL-8 by patient DCs in our experiments may be caused by a difference in maturation dynamics (43). The cause of this difference is unclear, but one could imagine some form of HCV-mediated imprinting in the infected precursor
cells (i.e. monocytes) leading to aberrations in differentiated cells.

Finally, the cytokine profile from the DCs may be confounded because all five patients whom we studied were previous non-responders to IFN-based therapy. It is unknown whether changes in cytokine secretion by DCs in patients with HCV infection are related to treatment outcome.

In contrast to our results, Longman et al. (44) have used PGE2 and TNF-α to generate functional monocyte-derived DCs from 13 chronic HCV patients. However, (i) they used a lower concentration of PGE2, a higher concentration of TNF-α and no IL-1β or IL-6, which may have influenced the form and function of the DCs, (ii) they used different assays to assess DC function and (iii) only four of 13 patients in their study were previous non-responders vs 10 of 13 patients in our study. Taken together, these differences make comparison of our results and Longman’s results difficult.

Why are DCs impaired in chronic HCV patients? Apart from HCV-specific impairments, there is no evidence that HCV patients have abnormal DC function. The impairments may be because of infection...
with HCV of DCs (10, 25, 26, 28, 45, 46) and/or their precursors in vivo. HCV core and NS3 inhibit DC maturation in vitro (12, 47, 48) and HCV core, NS3, NS5A and NS5B induce apoptosis in DCs (49). DC dysfunction in chronic HCV infection could then be a direct and/or downstream event. This would also explain why other groups observed impaired DC function in chronic HCV patients, but normal DC function after patients achieved SVR (9, 10). Another probability is that the inflammatory state per se in chronic HCV patients compromises DC maturation and/or function (50).

In summary, we have shown that DCs free from HCV RNA can be generated from monocytes obtained from chronic HCV patients. However, these DCs have an immature expression pattern of DC surface markers, and their function is abnormal, suggesting impaired ability of monocyte-derived DCs from chronic HCV patients to prime T<sub>1</sub>1 responses. The observed aberrations suggest another mechanism by which HCV interferes with innate immunity and ultimately cellular immunity.

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DCs from chronic HCV patients


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