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INTRODUCTION

Hepatitis C virus (HCV) infection is the cause of chronic hepatitis in more than 4 million people in the USA^[1]. Patients with chronic HCV infection experience a various severity of liver diseases ranging from mild hepatitis to cirrhosis and hepatocellular carcinoma. Liver cirrhosis that appears decades after the initial infection often predisposes to spontaneous bacterial peritonitis and sepsis^[2]. HCV is the most frequent indication for liver transplantation, and transplant recipients suffer from recurrent HCV and infections associated with immunosuppression^[3]. HCV, a blood borne pathogen, is frequently associated with human immunodeficiency virus (HIV) infection acquired through the same route of invasion^[4]. According to clinical data, HCV is not only the cause of morbidity due to liver disease, but it is also associated with other infections. A recent study by El-Sarag *et al*^[5] suggested that infections with other viral and bacterial pathogens were more common in HCV-infected patients who otherwise had no immunocompromise compared to non-HCV infected controls. The pathogens causing infections at a higher rate in HCV patients included cytomegalovirus, cryptococcus, *Mycobacterium tuberculosis* and those causing sexually transmitted diseases^[5]. It remains to be evaluated whether recognition of pathogens and/or mounting appropriate immune responses is impaired in HCV infection.

Bacterial and viral pathogens are recognized as “danger” signals by the family of Toll-like receptors (TLRs) that alert innate immunity against the invading pathogens^[6]. The known 11 mammalian TLRs have distinct extracellular domains but share common intracellular mechanisms of cell activation that culminate in production of inflammatory cytokines. Each TLR has specific ligands, which allow the host to sense a wide diversity of pathogens. TLR1, TLR6 and possibly TLR10 form heterodimers with TLR2 to recognize fungi (*Sarcomyces cerevisiae*, *Candida albicans*, and *Aspergillus fumigatus*), parasites (*Trypanosoma cruzi*), Gram positive bacteria-derived peptidoglycan (PGN) and bacterial lipoproteins, lipoarabinomannan from *Mycobacterium tuberculosis*, *Treponema pallidum*-derived glycolipid

Abstract

AIM: Hepatitis C virus often establishes chronic infections. Recent studies suggest that viral and bacterial infections are more common in HCV-infected patients compared to controls. Pathogens are recognized by Toll-like receptors (TLRs) to shape adaptive and innate immune responses.

METHODS: In this study, to assess the ability of HCV-infected host to recognize invading pathogens, we investigated Toll-like receptor expression in innate (monocytes) and adaptive (T cells) immune cells by real-time PCR.

RESULTS: We determined that RNA levels for TLRs 2, 6, 7, 8, 9 and 10 mRNA levels were upregulated in both monocytes and T cells in HCV-infected patients compared to controls. TLR4 was only upregulated in T lymphocytes, while TLR5 was selectively increased in monocytes of HCV-infected patients. MD-2, a TLR4 co-receptor, was increased in patients' monocytes and T cells while CD14 and MyD88 were increased only in monocytes.

CONCLUSION: Our data reveal novel details on TLR expression that likely relates to innate recognition of pathogens and immune defense in HCV-infected individuals.

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Key words: Hepatitis C virus; Toll-like receptors; T cells; Monocytes

Table 1 Patient characteristics

Parameter	Value
Gender (male/female)	12/2
Age (yr)	44 ± 12
Duration of disease	> 10 yr
	Unknown
	9
Viral load (by QUNT BDNA, IU/ml)	1 × 10 ⁷ ± 0.78 × 10 ⁷
Viral genotype	(by 1a)
PCR)	5
	1b
	4
	3a
	3
	4a
	2
Plasma SGOT (ALT) levels (IU/mL)	110 ± 86
Plasma SGPT (AST) levels (IU/mL)	102 ± 60

and lipoteichoic acid^[6,7]. TLR2 can also recognize certain viruses such as cytomegalovirus, measles virus and core and NS3 proteins of HCV^[8-10]. TLR3 recognizes poly(I-C) and double-stranded viral RNA, whereas TLR4 agonists include Gram-negative bacterial LPS, respiratory syncytial virus, *Cryptococcus neoformans* and the plant product Taxol^[6,7,11]. Bacterial flagellin has been identified as a TLR5 ligand, synthetic components imiquimod and resiquimod 848 and single stranded RNA stimulate TLR 7 and 8, while unmethylated CpG-containing DNA and herpes simplex virus have been identified as TLR9 agonists^[6,7,12].

The presence of TLRs was originally discovered in innate immune cells^[13]. Monocytes express all TLRs of which TLRs 1, 2, and 4 are present at high levels^[14]. Latest research, however, indicates that TLR expression is not restricted to innate immune cells and TLRs can be detected in adaptive immune cells as well as in parenchymal cells. In particular, T cells express all TLRs at low levels with the exception of TLR5, which is present abundantly^[14, 15].

In order to dissect the ability of HCV-infected host to respond to invading pathogens, we investigated Toll-like receptor expression in innate (monocytes) and adaptive (T cells) immune cells. We determined that TLR2, 7, 8, 9 and 10 were upregulated in both immune compartments in HCV-infected patients compared to controls. In addition, TLR4 was upregulated in lymphocytes and TLR6 was upregulated in monocytes only. Our data reveal new insights into immune defense of HCV-infected individuals.

MATERIALS AND METHODS

Blood donors

Healthy individuals (controls, *n* = 14) and treatment-naïve patients chronically infected with hepatitis C virus (HCV patients, *n* = 14) were enrolled in the study. All individuals were free of acute infections. The study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School and informed consent was obtained. The clinical characteristics of the patients are detailed in Table 1. The blood was collected from cubital vein with anti-coagulant (heparin sodium) and processed immediately. Controls and patients were matched for age and gender where possible.

Cells

Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll gradient. In order

Table 2 Real-time PCR primers

Primer identification	Sequence
TLR1	Forward 5'-GGG TCA GCT GGA CTT CAG AG-3'
	Reverse 5'-AAA ATC CAA ATG CAG GAA CG-3'
TLR2	Forward 5'-GCC TCT CCA AGG AAG AAT CC-3'
	Reverse 5'-TCC TGT TGT TGG ACA GGT CA-3'
TLR3	Forward 5'-GTG CCA GAA ACT TCC CAT GT-3'
	Reverse 5'-TCC AGC TGA ACC TGA GTT CC-3'
TLR4	Forward 5'-AAG CCG AAA GGT GAT TGT TG-3'
	Reverse 5'-CTG AGC AGG GTC TTC TCC AC-3'
TLR5	Forward 5'-TTG CAT CCA GAT GCT TTT CA-3'
	Reverse 5'-TTC AAC TTC CCA AAT GAA GGA-3'
TLR6	Forward 5'-GAA CAT GAT TCT GCC TGG GT-3'
	Reverse 5'-GCT GTT CTG TGG AAT GGG TT-3'
TLR7	Forward 5'-AAT GTC ACA GCC GTC CCT AC-3'
	Reverse 5'-GCG CAT CAA AAG CAT TTA CA-3'
TLR8	Forward 5'-TGT GAT GGT GGT GTC TCA AT-3'
	Reverse 5'-ATG CCC CAG AGG CTA TTT CT-3'
TLR9	Forward 5'-ATT CTG ACT TTG CCC ACC TG-3'
	Reverse 5'-GCT GAG GGA CAG GGA TAT GA-3'
TLR10	Forward 5'-GGC CAG AAA CTG TGG TCA AT-3'
	Reverse 5'-AAA TGA CTG CAT CCA GGG AG-3'
MyD88	Forward 5'-GAG CGT TTC GAT GCC TTC AT-3'
	Reverse 5'-CGG ATC ATC TCC TGC ACA AA-3'
MD2	Forward 5'-ATT CCA AGG AGA GAT TTA AAG CAA TT-3'
	Reverse 5'-CAG ATC CTC GGC AAA TAA CTT CTT-3'
CD14	Forward 5'-CGC TCC GAG ATG CAT GTG-3'
	Reverse 5'-TTG GCT GGC AGT CCT TTA GG-3'

to separate monocytes from lymphocytes, the PBMCs were plated (10⁷/well in 2 mL of RPMI1640 media supplemented with 10 % FBS) in 6 well plates (Corning, Corning, NY) and incubated at 37 °C in 50 ml/L CO₂ atmosphere for 3 h. Monocytes were separated based on their adherence to plastic and the purity of the population, based on CD14 expression, was greater than 95% as determined by flow cytometry (data not shown). Non-adherent cells were washed and T lymphocytes were purified using T cell negative isolation kit (DynaL Biotech Inc, Lake Success, NY), as manufacturer recommended. Briefly, non-adherent cells were incubated with a cocktail of antibodies for CD14, CD16a, CD16b, CD56, HLA class II DR/DP and CD235A, followed by incubation with depletion magnetic Dynabeads. The non-T cells bound to the Dynabeads and were separated in a strong magnetic field while T cells were washed and subjected to RNA extraction.

Real-time PCR

Total cellular RNA was extracted using RNeasy kits (Qiagen, Valencia, CA), according to manufacturer's recommendations. All samples were co-processed to eliminate technical variations. Equal amounts of RNA from controls and HCV-infected patients were analyzed. Reverse transcription of 1 µg of RNA into cDNA was performed using reverse transcription System (Promega, Madison, WI). The real-time PCR primers were synthesized by IDT (Coralville, Iowa), except for 18S (Quantum RNA Classic II 18S Internal Standard, Ambion, Austin, TX). The real-time PCR was performed using the ICycler (Biorad Laboratories, Hercules, CA). The PCR primers are described in Table 2. The reaction mixture contained 12.5 µL qPCR

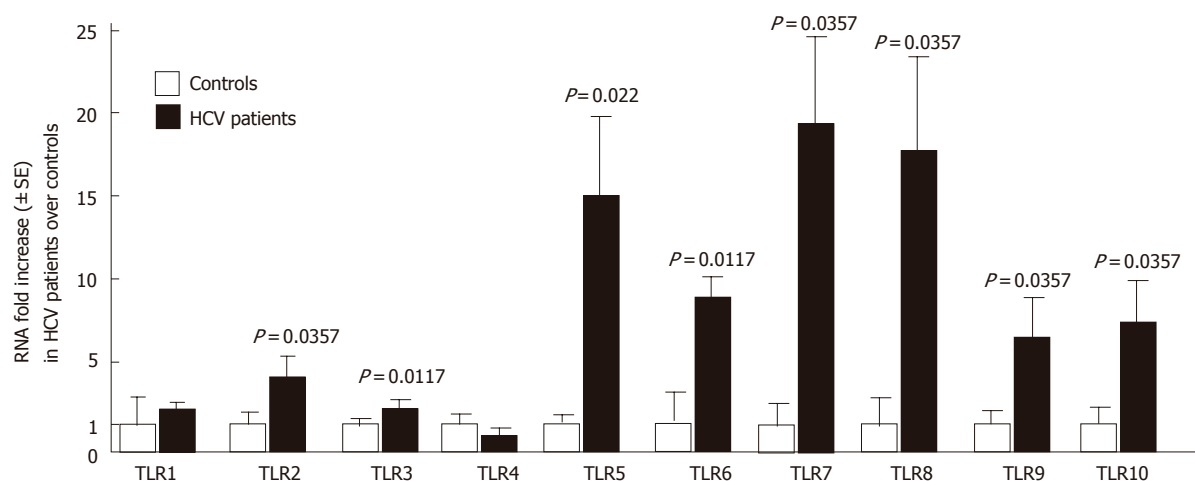


Figure 1 Higher TLRs 2, 5, 6, 7, 8, 9 and 10 RNA expression by monocytes of HCV-infected patients compared to controls. RNA was purified from adherence-isolated monocytes and real-time PCR for TLRs 1-10 and 18 S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual, TLR-coding RNA expression was normalized to corresponding 18 S RNA. Results are expressed as fold increase of each TLR in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

MasterMix Plus with 2 x SYBR Green (Eurogentec, Seraing, Belgium), 0.5 $\mu\text{mol/L}$ of forward and reverse primer and 1 μL of cDNA (corresponding to 50 ng of RNA) in a total volume of 25 μL . All amplifications and detections were carried out in a BioRad optical 96-well reaction plate with optical tape. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by dsDNA-binding SYBR Green. After the PCR was performed, a dissociation/melting curve was constructed in the range of 55°C to 95°C. Data were analyzed using the Biorad ICycler software. As a first step in data analysis 18S was used for normalization of all experiments (normalizer) and it was closely compared between controls and HCV patients. For second step in data analysis we used comparative Ct method ($\Delta\Delta\text{Ct}$ method) with the following formula: $\Delta\text{Ct} = \text{Ct}(\text{target, TLR}) - \text{Ct}(\text{normalizer, 18S})$. The comparative $\Delta\Delta\text{Ct}$ calculation involved finding the difference between ΔCt of HCV patient and the mean value of the ΔCt from normals for each analyzed molecule. Fold increase in the expression of specific mRNA in HCV patients compared to normal controls was calculated as $2^{-(\Delta\Delta\text{Ct})}$.

Statistical analysis

The non-parametric Wilcoxon test from StatView on a G4 Machintosh platform (Cupertino, CA) was employed to determine the statistical difference between controls and HCV patients.

RESULTS

HCV-infected patient selection and their characteristics

Our study population included patients with chronic HCV infection but without cirrhosis or cancer, thus, HCV infection-specific imprints on the immune system were present but cirrhosis or cancer-related non-specific immune alterations could be excluded. All patients in the study had viremia and elevated levels of liver transaminases consistent with chronic hepatitis. Patients

infected with different viral genotypes (1A, 1B, 3A and 4A) were included, as viral genotype could affect host immune response^[16]. Our patients had never been treated with anti-viral therapy, thus possible influence of therapy on TLR expression was eliminated. This is important because Ribavirin was shown to augment signaling in TLR-transfected cells^[17].

Monocytes of HCV-infected patients express higher levels of TLRs 2, 3, 5, 6, 7, 8, 9 and 10 RNA compared to controls

Chronic HCV infection is associated with increased serum levels of inflammatory cytokines^[18]. Blood monocytes and tissue macrophages are major sources of these cytokines. We recently reported increased monocyte production of TNF α in patients with chronic HCV infection^[10]. In this study, we used real-time PCR to evaluate TLR expression in monocytes of HCV-infected patients and healthy controls. Monocytes both from controls and HCV-infected patients expressed TLRs 1-10 (Figure 1), as expected based on previous studies^[14]. We found that monocytes from patients with chronic HCV infection expressed significantly higher levels of RNA coding for TLR2, TLR5, TLR6 and TLR10. Different TLRs reside at different locations in the cell. TLRs 1, 2, 4, 5, 6 and 10 are expressed mostly on the surface of the cellular membrane, while TLRs 3, 7, 8 and 9 are localized inside the cells^[6,7]. Surprisingly, we found that not only some outer cellular membrane-associated TLRs, but also the intracellularly localized TLRs, TLRs 7, 8 and 9, were upregulated in HCV patients' monocytes. The level of TLR3 mRNA was also significantly higher in HCV-infected patients compared to controls.

Ligand recognition and signaling of TLR2 and TLR4 is augmented by co-receptors^[6,7]. Thus, we further analyzed the expression of TLR co-receptors and adaptors in both controls and HCV-infected patients. We found that the mRNA level of CD14, a co-receptor for TLR2 and TLR4, was upregulated in HCV patients compared to controls (Figure 2). In addition to CD14, MD-2 is another accessory protein of the TLR4 that is necessary

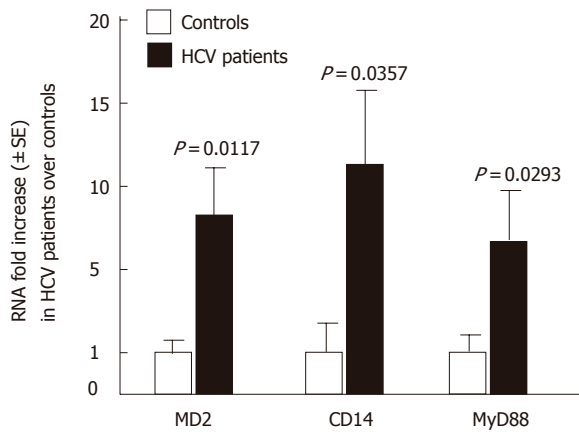


Figure 2 Monocytes of HCV-infected patients express higher levels of TLR co-receptors MD2 and CD14, and TLR adaptor MyD88 compared to controls. RNA was purified from adherence-isolated monocytes and real-time PCR for MD2, CD14, MyD88 and 18 S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual protein of interest-coding RNA expression was normalized to corresponding 18 S RNA. Results are expressed as fold increase of each protein of interest in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

for assembling the TLR4-containing receptor complex to sense low quantities of lipopolysaccharide^[19]. We found that MD2 mRNA was expressed at significantly higher levels in monocytes of HCV infected patients compared to controls. The mRNA for MyD88, an intracellular adaptor molecule that is recruited to the intracellular domain of TLRs 2, 4, and 9 upon engagement with specific ligands, was also higher in patients compared to controls. These results suggest that increased RNA expression of various TLRs and their co-receptors may predispose monocytes to increased TLR-mediated activation in chronic HCV.

Lymphocytes of HCV-infected patients express higher levels of TLRs compared to controls

We next investigated the expression of TLRs in CD3+ T cells of the adaptive immune cells. T cells from controls and HCV infected patients expressed mRNA for TLRs1-10 (Figure 3) as expected based on previous publications^[14,15]. There was no CD14 mRNA expressed in T lymphocytes of either controls or HCV-infected patients, confirming the purity of the cell population (data not shown). We found that levels of RNA coding for membrane-localized TLR1, 2, 4, 6 and 10 as well as the TLR4 co-receptor, MD2, were significantly higher in HCV infected patients compared to controls. No differences in levels of RNA coding for MyD88 or TLR3 and TLR5 (Figure 3) between controls and patients were identified. In contrast, the RNA levels of intracellularly localized TLRs 7, 8 and 9 were significantly higher in T lymphocytes of HCV infected patients compared to controls.

DISCUSSION

Monocytes and lymphocytes, respectively, are representative populations of innate and adaptive immunity and their cooperation is required to recognize, limit and eliminate invading microbes^[20]. Here we show that patients infected with HCV have a unique pattern of TLR expression in

both the innate and adaptive immune compartments and have elevated RNA expression of both membrane-associated and intracellularly localized TLRs. Recent reports demonstrate that HCV-infected patients have a higher prevalence of infections with cytomegalovirus, Cryptococcus, Mycobacterium tuberculosis and sexually transmitted diseases compared to controls^[5]. Recognition of these pathogens requires TLR-mediated signals. For example, sensing of Cryptococcus by the innate immune system requires TLR2, TLR4, MyD88 and CD14^[21], while TLR2 and TLR4 are instrumental in the host's response against Mycobacteria^[22]. The pathogens implicated in sexually-transmitted diseases, found more often in HCV-infected patients, are also recognized by TLR. Chlamydia elicits an unusual set of inflammatory responses via TLR2 and TLR4 *in vivo*, and TLR2 is essential for development of oviduct pathology in chlamydial genital tract infection^[23], while *Neisseria gonorrhoeae* stimulates cytokine release and NF- κ B activation in epithelial cells in a TLR2-dependent manner^[24]. In addition, some TLRs may play a protective or even therapeutic role in defense against sexual disease pathogens: TLR3 agonists protect against genital herpes infection^[25] and Imiquimod, a TLR7&8 ligand and the first FDA-approved imidazoquinoline, has been approved for the therapy of genital warts^[26]. Increased expression of TLR2 and TLR4 is a bad prognostic factor in patients with sepsis^[27], while low TLR expression may protect the host against excessive inflammation and tissue damages^[28], thus, differential expression of TLRs in HCV infected patients may contribute to susceptibility to infections as well as to the underlying disease progression and this remains to be determined.

The observation of increased expression of TLR2 in patients was not totally unexpected, since chronic HCV infection is associated with elevated levels of circulating LPS, which upregulates the expression of TLR2 in different cell types, including monocytes^[21-23]. We have previously shown that HCV core and non-structural protein 3 (NS3), activate cells through TLR2^[10]. We found elevated levels of the TLR co-receptors, CD14 and MD2, and adaptor molecule MyD88, in HCV-infected patients compared to controls, suggesting that MyD88-mediated TLR signal transduction may also be affected in patients. Indeed, we have previously found that monocytes from HCV infected patients are hyper-responsive to stimulation with TLR4 and TLR2 ligands^[10,32]. Although we did not find a role for CD14 in TLR2-mediated cell activation by HCV-derived core and NS3 proteins (unpublished data), increased CD14 expression may be relevant to cell activation mediated by other TLR2 and TLR4 ligands, as previously reported for LPS, lipoteichoic acid and even viruses^[9].

Intracellularly-localized TLR 3, 7, 8 and 9 are of specific interest in patients with chronic viral infections since these receptors can recognize virus-derived molecular patterns^[6,7]. We found that TLR3 expression was higher in monocytes of HCV-infected patients compared to controls. TLR3 is a sensor for double-stranded RNA and it has been implicated in pathogenesis of viral infections^[33,34]. Synthetic RNA compounds, such as polyI:C activate cells expressing TLR3^[35]. In a recent study by Edelman *et al.*^[36] the role of TLR3 was investigated in four differ-

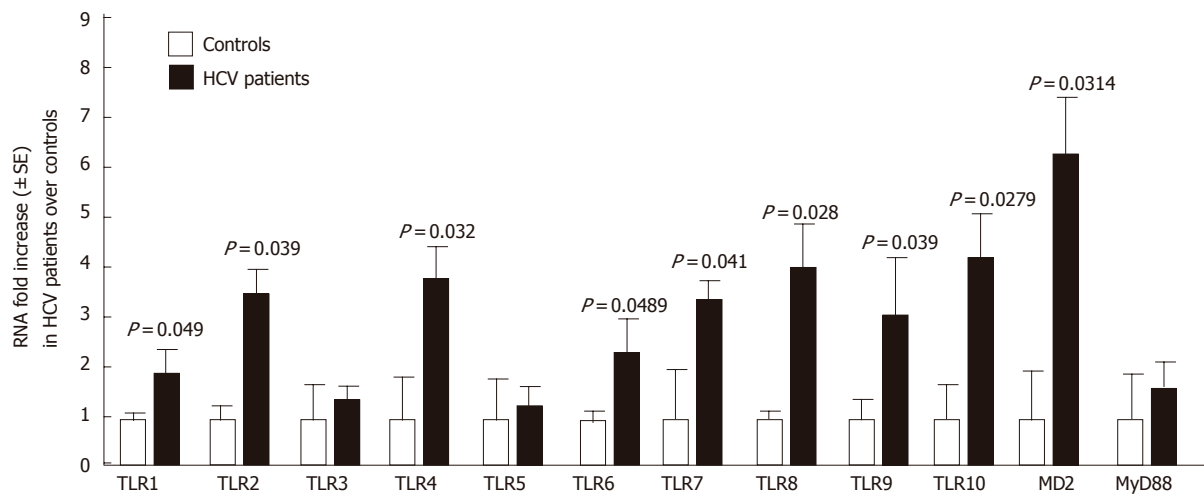


Figure 3 Higher TLRs 1, 2, 4, 6, 7, 8, 9 and 10 and MD2 RNA expression by lymphocytes of HCV-infected patients compared to controls. RNA was isolated from lymphocytes and real-time PCR for TLRs 1-10, MD2 and 18S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual, TLR- or MD2-coding RNA expression was normalized to corresponding 18S RNA. Results are expressed as fold increase of each TLR or MD2 in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

ent infectious viral models [lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and reovirus in TLR3^{-/-} mice]. The investigators found that TLR3 is not always required for the generation of effective antiviral responses, as the absence of TLR3 did not alter either viral pathogenesis or host's generation of adaptive antiviral responses to those viruses. Interestingly, intracellular transduction of poly(I:C) initiates activation of an IFN response in a TLR3-independent manner, thus limiting the role of TLR3 in the IFN pathway^[55]. A recent report by Li *et al*^[54] indicates that HCV may use TLR3 pathway to evade immune surveillance via HCV NS3/4A protease-mediated cleavage of the TLR3 adaptor protein TRIF. Furthermore, HCV NS3/4A interferes with retinoic acid-inducible gene-I (RIG-I), a key factor in TRIF-independent signaling^[57]. Our finding of increased TLR3 mRNA levels in monocytes of HCV patients awaits further investigation. It is tempting to speculate that HCV RNA may trigger cellular activation via TLR3, and indeed, all our patients had high levels of viremia, however no clear relationship between HCV RNA levels and TLR3 expression has been proven to date.

TLR7 and TLR8 are stimulated by synthetic compounds imiquimod and resiquimod 848^[6,7,55]. In a recent publication, Lund *et al*^[12] showed that TLR7 recognizes the single-stranded RNA viruses, vesicular stomatitis virus and influenza virus. The recognition of these viruses by plasmacytoid dendritic cells and B cells through TLR7 results in cellular activation and the production of cytokines. However, the specific role of TLR7 and TLR8 in innate immune response to HCV is yet to be understood. HCV is a single stranded RNA virus, thus, it could theoretically act as a ligand for TLR7. Our data show that TLR7 mRNA is significantly upregulated in HCV patients compared to controls, and all patients had detectable levels of HCV RNA in their plasma, thus suggesting that TLR7 may be implicated in the pathogenesis of HCV infection.

We found that both monocytes and T cells of HCV-infected patients expressed elevated levels of TLR9

mRNA compared to controls. CpG-containing DNA acts as a stimulatory ligand for TLR9^[6,7,29,35]. TLR9 is regulated at the transcriptional level by multiple nuclear regulatory factors, co-repressors and co-activators, including CREB1, Ets2, Elf1, Elk1, and C/EBP and HCV may regulate these transcription factors^[38,39]. Toll-like receptors 9 and 3 are essential components of innate immune defense against cytomegalovirus infection in mice^[40]. While we found that TLR9 mRNA was upregulated in patients, others have shown that infections with cytomegalovirus are more frequent in HCV-infected patients compared to controls^[5]. It remains to be elucidated if the increased TLR9 RNA levels in monocytes or T cells have a role in increased susceptibility to infections with CMV, as seen in HCV patients.

To date little is known about the role of TLR expression in T cells of the adaptive immune compartment. Caramalho *et al*^[41] recently reported that LPS, a TLR4 ligand, promotes survival of activated CD4⁺ cells and stimulates regulatory T cell (T reg) activity. Here we found that TLR4 mRNA expression was higher in HCV-infected patients compared to controls only in lymphocytes, but not in monocytes. It is tempting to speculate that elevated levels of TLR4 and its co-receptor, MD-2, in patients' T cells may provide ongoing activation in the presence of serum LPS found in chronic HCV and provide preferential survival signals for T regs^[30]. Recent data suggest a role for T regs in immune alterations associated with chronic HCV. Cabrera *et al*^[42] reported that Tregs appear to play a role in viral persistence by suppressing HCV-specific T cell responses in a cell-cell contact manner, while MacDonald *et al*^[43] showed that Tregs could be induced against the same epitopes on the HCV core protein.

A limitation of our study is that it did not distinguish between TLR expression in CD4⁺ and CD8⁺ T cells. Differential CD4⁺ and CD8⁺ T-cell responsiveness in hepatitis C virus infection has been reported, thus, leading to the hypothesis that if TLRs are of any functional role in T cells, there may be differences in TLR expression between CD4⁺ and CD8⁺ T cells in HCV-infected patients. Due to

the presence of anti-CD56 mAbs in the separation cocktail, we also lost CD56⁺ T cells, known as gamma delta, with an important role in HCV infection^[44].

In conclusion, we showed that patients with chronic HCV infection express elevated levels of selected TLRs in both adaptive and innate compartments of the immune system. Our data may suggest additional therapeutic targets.

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