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Viral and host factors induce macrophage activation and loss of Toll Like Receptor tolerance in chronic HCV infection

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Abstract

Background&Aims—Persistent inflammation contributes to progression of liver damage in chronic HCV (cHCV) infection. Repeated exposure to Toll like receptor (TLR) ligands results in tolerance, a protective mechanism aimed at limiting inflammation.

Methods—Monocytes/macrophages were repeatedly stimulated via pro-inflammatory cytokine-inducing TLRs and evaluated for activation markers.

Results—Unlike monocytes (Mo) of controls or patients with non-alcoholic steatohepatitis, the Mo of cHCV patients were hyper-responsive and failed to show homo- or hetero-tolerance to TLR ligands, manifested by elevated TNF α production. Serum levels of IFN γ , endotoxin (TLR4 ligand) and HCV core protein (TLR2 ligand) were elevated in cHCV patients suggesting potential mechanisms for *in vivo* monocyte pre-activation. Treatment of normal monocytes with IFN γ resulted in loss of tolerance to LPS or HCV core protein. Further, we found increased levels of MyD88-IRAK1 complexes and NF κ B activity both in monocytes of cHCV patients and in normal monocytes that lost TLR tolerance after IFN γ +LPS pretreatment. *In vitro* differentiation of TLR tolerant cHCV monocytes into macrophages restored their capacity to exhibit TLR tolerance to LPS and HCV core protein and this could be reversed by administration of IFN γ . cHCV patients exhibited increased TNF α in the circulation and in the liver. In cHCV livers we found Kupffer cell/macrophage activation indicated by increased CD163 and CD33 expression.

Conclusions—We identified that host-derived factors (IFN γ and endotoxin) and viral factors (HCV core protein) act in tandem to induce and maintain monocyte/macrophage activation, thus favoring persistent inflammation in patients with cHCV infection.

Keywords

MyD88; IFN γ ; HCV core protein; LPS; monocytes; Kupffer cells; NF κ B; TNF α ; inflammation

Introduction

Hepatitis C virus (HCV) infection leads to chronic disease in ~70% of infected patients (1). Many of the HCV-associated symptoms as well as progression to liver fibrosis are caused by

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persistent inflammation. Over-production of pro-inflammatory cytokines, in particular TNF α , was reported in patients with chronic HCV infection (2,3), however, the mechanisms leading to increased TNF α production and persistent inflammation during chronic HCV infection are yet to be fully understood.

Innate immunity plays a key role in the outcome of infections. Immune cells employ toll-like receptors (TLRs) to recognize specific pathogen-associated molecular patterns (4–7), such as dsRNA (TLR3), ssRNA (TLR7/8) that are present in the HCV life cycle (5), and lipopolysaccharide (LPS) (TLR4). TLR2 is activated by multiple ligands including HCV core protein (4,6,7). Upon engagement with specific ligands most TLRs, except for TLR3, recruit MyD88, IL-1 receptor-associated kinases (IRAK) and TNF receptor-associated factor 6 (TRAF6), and trigger NF κ B and MAPK activation pathways to results in production of pro-inflammatory cytokines, including TNF α (4,6). Recently cross-tolerance between TLRs has been documented (8–10). Decreased production of TNF α in response to challenge with a TLR ligand with which the cells were pre-treated has been defined as “homotolerance”, while decreased activation to a TLR ligand that does not share homology with the pre-treatment ligand is called “heterotolerance”(8). TLR tolerance plays an important role in protection from hyperactivation of the immune system and can be overcome by conditioning of innate immune cells with cytokines, including interferons and growth factors (8–10).

Kupffer cells (KCs) are liver resident macrophages and a part of the body-wide distributed monocyte/macrophage system (11). KCs express TLRs and respond to pathogens with inflammatory cytokines, including TNF α (12). Activated KCs play a pivotal role in triggering and maintaining inflammation during alcoholic-and non-alcoholic liver diseases (13,14). During cHCV infection Kupffer cells exhibit an activated phenotype (15,16); however they do not support HCV replication *in vitro* (17). The role of Kupffer cells in inflammation during chronic infection with HCV is yet to be fully understood.

Here we hypothesized that activation of macrophages, including Kupffer cells, may favor the persistent inflammation in patients with chronic HCV infection. We identified that host-derived factors (IFN γ and endotoxin) and viral factors (HCV core protein) act in tandem to induce and maintain monocyte/macrophage activation, thus favoring persistent inflammation in patients with chronic HCV infection.

Materials and Methods

Reagents

LPS was from List Biological Laboratories (Campbell, CA), peptidoglycan (PGN) from Fluka (Milwaukee, WI), Pam₂CSK₄ and Pam₃CSK₄ from EMC Microcollections (Germany), Gardiquimod from Invivogen (San Diego, CA), fetal calf serum (FCS) from HyClone (Logan, UT), RPMI1640 from Gibco (Grand Island, NY), recombinant HCV core protein (genotype 1A aa 2–192) from Biodesign (Saco, MN).

Patients and Cells

The study was approved by the Committee for the Protection of Human Subjects in Research at UMass. Healthy controls (n=24), treatment-naïve patients with chronic HCV infection (cHCV patients, n=30) and patients with non-alcoholic steatohepatitis (NASH, n=6) were enrolled in our study after informed consent was obtained; patient’s characteristics are detailed in table 1.

Monocytes were separated from blood by centrifugation in Ficoll gradient and adherence to plastic as we previously described (6). Macrophages were differentiated *in vitro* from

monocytes for 8 days in RPMI1640 with 1% non-essential aminoacids and 18% pooled normal human platelet-free serum.

For Kupffer cells isolation, C57Bl6 mice received anesthesia with Ketamine (100mg/kg) and Xylazine (10mg/kg) and the livers were perfused with saline for 5 minutes followed by digestion with liberase (20mg/L) for 5 minutes. The livers were excised, minced and further digested *in vitro* for 30min at 37°C. The hepatocytes were separated by centrifugation for 5min at 300xg, while the non-hepatocyte content was loaded on the top of the 50%-25% Percoll gradient and centrifuged 60 minutes at 800xg. The inter-cushions fraction was washed and adhered to plastic in DMEM+5%FBS; the non-adherent fraction was washed and the adherent population was adjusted to 2×10^6 /ml in DMEM+10%FBS.

Quantification of cytokines, endotoxin, and HCV core protein

The cytokines were quantified using specific ELISA (BD Bioscience, San Jose, California), HCV core protein with Ortho HCV core antigen ELISA (Wako Chemicals USA, Richmond, VA) and endotoxin by Lymulus Amebocyte Lysate assay (Lonza Group Ltd, Switzerland).

Western blot analysis and Immunoprecipitation

Total cellular proteins (25µg) were separated in SDS-PAGE, transferred to nitrocellulose membrane and immuno-blotted with indicated primary followed by horseradish peroxidase-conjugated secondary antibodies. The densitometric analyzed of the specific bands was performed with UVP system and LabWork 4.0 program (UVP BioImaging Systems). For immunoprecipitation, equal amounts of cellular proteins were incubated with anti-MyD88 antibody (Santa Cruz) for 16hrs at +4°C. The immunoprecipitated complexes were purified using Catch&Release system (Upstate, Charlottesville, VA) and subjected to western blot analysis as described above.

Electrophoretic mobility shift assay (EMSA)

Subcellular extracts and EMSA were performed as previously described (6). The densitometric analysis was performed using GDS-800 system and Labwork 4.0 program.

RNA Isolation and PCR analysis

RNA was isolated with RNeasy Kit (Qiagen, Valencia, CA). One µg of RNA was transcribed to cDNA in 20µl reaction volume. For real-time PCR, the SYBR Green Mix (Eurogentec, San Diego, CA) was combined with primers (sequences are shown in Table 2) and 1µl of cDNA and amplified using iCycler (Bio-Rad, Hercules, CA). Gene amplification was quantified based on SYBR Green fluorescence with iCycler software and analyzed using comparative $\Delta\Delta C_t$ method using 18S as housekeeping control. For regular PCR, the products were separated in 1% agarose gels and the intensities of the bands were analyzed by densitometric analysis as for EMSA.

Statistical analysis

The Students t-test and Wilcoxon non-parametric analysis in StatView (SAS Inst, Cary, NC) were employed. A value of $p < 0.05$ was considered significant.

Results

Monocytes of cHCV patients lack tolerance to TNF α -inducing TLR ligands

We have previously reported that monocytes of cHCV patients produced significantly higher levels of TNF α both at baseline and upon stimulation with TLR4 and TLR2 ligands compared to controls (6). Production of TNF α by immune cells contributes to the efficient control of

invading pathogens, while excessive and uncontrolled production may lead to systemic chronic inflammation (2,3). Tolerance to TLR ligands is one of the protective mechanisms against chronic inflammation (10). Considering the presence of chronic inflammation in cHCV patients, we hypothesized that excessive production of TNF α in monocytes may represent loss of tolerance to TLR ligands. Monocytes of controls and HCV patients were primed *in vitro* with LPS, a TLR4 ligand, and re-stimulated with LPS to assess “homotolerance”. In agreement with previous reports (8,9), we found that LPS priming lead to low TNF α production in response to a re-challenge with LPS in normal monocytes (Fig 1A). However, homo-tolerance to TLR4 ligand was not found in monocytes from HCV patients (Fig 1B). We further identified that pretreatment with LPS induced hetero-tolerance to subsequent stimulation with TLR2 (PGN), TLR2/TLR6 (PAM₃CSK₄), TLR2/TLR1 (PAM₂CSK), TLR3 (poly I:C) and TLR7/8 (Gardiquimod) ligands in controls (Fig 2A), and in patients with liver inflammation due to non-viral non-alcoholic steatohepatitis (NASH) (Fig 2C) but not in HCV-infected patients (Fig 2B). This data indicated that monocytes of HCV-infected patients have lost TLR tolerance.

HCV-infected patients exhibit elevated serum levels of IFN γ , endotoxin and HCV core protein

To dissect the mechanisms of loss of tolerance in monocytes of cHCV patients, we analyzed their serum in search of immunomodulatory molecules. We identified moderate but significantly higher levels of TNF α (data not shown) and IFN γ in the plasma of cHCV patients compared to controls (Fig 3A). IFN γ is among the most potent agents that may lead to abrogation of TLR-mediated tolerance (8,9). The plasma levels of IFN γ were biologically active *in vivo*, as indicated by increased expression of IFN γ -dependent genes (CXCL9, CXCL10, CXCL11, and RIG-I) in monocytes of patients compared to controls (Fig 3B). We also found increased levels of circulating endotoxin, a TLR4 ligand, in the plasma of HCV-infected patients compared to controls (Fig 3C). Finally, cHCV patients but not controls had detectable plasma levels of HCV core protein (Fig 3D), a potent immunomodulator (6,18). No correlation between HCV core protein and IFN γ in plasma was identified (data not shown).

IFN γ , endotoxin and HCV core protein modulate monocyte activation towards loss of TLR tolerance

Previous studies demonstrated that HCV core protein activates monocytes via TLR2 (6,7). Here we show that repeated stimulations with HCV core protein, similar to other TLR2 ligands (Fig 2), induced tolerance in normal monocytes (Fig 4A). The monocytes of HCV patients produced more TNF α compared to normal cells in response to HCV core protein (Fig 4A). However, in contrast to normals and to NASH patients, the monocytes from HCV-infected patients failed to develop tolerance upon repeated stimulations with HCV core protein (Fig 4A). Based on the finding of increased IFN γ levels in the peripheral circulation of cHCV patients (Fig 3A) and previous reports suggesting the role of IFN γ in loss of tolerance to TLR ligands (8–10), we further hypothesized that IFN γ may play a role in increased TNF α production and loss of tolerance to TLR ligands in cHCV infection. Both IFN γ and LPS alone, in amounts comparable to levels detected in the plasma of cHCV patients, induced low levels of TNF α in normal monocytes (data not shown). Further, combined IFN γ +LPS stimulation induced higher levels of TNF α compared to LPS alone (data not shown). Interestingly, when the *in vivo*-detected levels of endotoxin were used to pre-treat normal monocytes followed by stimulation with a higher dose of LPS, TNF α induction was inhibited, suggestive of tolerance (Fig 4B). Further, *in vivo*-detected levels of IFN γ could overcome the LPS tolerance (Fig 4B), and reverse both HCV core protein-induced homo-tolerance (Fig 4C) and LPS-rendered hetero-tolerance to HCV core protein (Fig 4D) in normal monocytes. These data suggest that HCV core, LPS and IFN γ favor loss of TLR tolerance and contribute to excessive production of TNF α in monocytes.

TLR plus IFN γ condition elevated NF κ B activation and upregulation of MyD88/IRAK1 complexes

Based on the findings of increased levels of both endotoxin and IFN γ in plasma of the cHCV patients (Fig 3) we hypothesized that TLR signaling pathways may also be amplified in cHCV patients. Activation of the NF κ B complex is instrumental in TLR-induced TNF α production (19,20). We found that pre-treatment with IFN γ enhanced LPS-induced NF κ B activity in normal monocytes (Fig 5A) consistent with increased amounts of TNF α produced in response to LPS+IFN γ -stimulation (Fig 4B). Similar to LPS+IFN γ -treated controls, the freshly isolated monocytes of cHCV patients exhibited increased levels of NF κ B activity (Fig 5B). Upon ligand engagement most TLRs recruit MyD88 and IRAK1 to initiate activation of the NF κ B pathway (4). Persistent NF κ B activation is favored by sustained presence of the MyD88/IRAK complexes (9). Immunoprecipitation approach identified that LPS triggered formation of MyD88/IRAK1 complexes (Fig 5C), while LPS+IFN γ -treated cells showed elevated level of MyD88/IRAK1 complexes compared to IFN γ - or LPS-treated monocytes (Fig 5C). Similar to *in vitro* LPS+IFN γ -stimulated controls, we identified significantly higher levels of MyD88/IRAK complexes in freshly isolated monocytes of cHCV patients compared to controls (Fig 5D). Together, these data suggested that the monocytes of cHCV patients exhibit molecular signatures of impaired TLR tolerance indicated by the presence of *in vivo* pre-formed MyD88/IRAK1 complexes, persistent activation of NF κ B, and increased production of TNF α . Further, the loss of TLR tolerance seen in cHCV monocytes could be reproduced *in vitro* by conditioning of normal monocytes with IFN γ and endotoxin.

The resistance to TLR tolerance in cHCV patients is reversible

Since monocytes are precursors for tissue macrophages, such as Kupffer cells (11), we differentiated monocytes *in vitro* with or without endotoxin, IFN γ or HCV core protein in order to identify if the loss of tolerance to TLR ligands seen in monocytes of cHCV patients is also a feature of macrophages. Similar to monocytes, normal macrophages responded with production of TNF α and showed both homo- and hetero-tolerance to endotoxin (Fig 6A) and HCV core protein (Fig 6B, C). However, IFN γ prevented the development of tolerance when added during macrophage differentiation and the first stimulation (Fig 6A). Surprisingly, *in vitro* differentiation of cHCV monocytes to macrophages restored their TLR tolerance: unlike freshly isolated cHCV monocytes (Fig 1B and Fig 2B), *in vitro* differentiated cHCV macrophages produced normal TNF α levels and showed tolerance to LPS (Fig 6A) and HCV core protein (Fig 6B, C). To approximate the *in vivo* conditions of the cHCV-infected host, we differentiated macrophages *in vitro* in the presence of IFN γ . Similar to control macrophages, IFN γ was capable of breaking both homo- and hetero-tolerance to LPS (Fig 6A) and to HCV core protein (Fig 6B, C) in cHCV macrophages, thus closely reproducing the lack of tolerance to TLR ligands seen in monocytes of cHCV patients (Fig 2B). These data suggested that the loss of tolerance to TLR ligands seen in cHCV monocytes is caused by *in vivo* environment and it is reversible; however it can be re-induced by modulatory agents, such as IFN γ , endotoxin and HCV core protein.

Patients with cHCV infection show evidence of macrophage activation in the liver

Finally, based on the activated phenotype of circulating cHCV monocytes and the stimulatory effect of IFN γ on macrophages, we hypothesized that the liver macrophages, Kupffer cells, may be activated *in vivo* during cHCV infection. Here we show that TNF α mRNA levels were significantly increased in livers of cHCV patients (Fig 7A). Kupffer cells produce TNF α and express CD163 and CD33, which are increased upon activation (16,17,21). We identified that CD163 (Fig 7B) and CD33 (Fig 7C) RNA levels were elevated in livers of cHCV patients compared to controls, suggesting Kupffer cell activation. We further employed mouse KCs to evaluate the relevance of the TLR tolerance. As shown in figure 7D, one-time *in vivo* encounter

with LPS renders KCs tolerant to either LPS or HCV core protein, similar to normal monocytes (Fig 1A and 2A). However, Kupffer cells exposed to LPS *in vivo* at multiple occasions during prolonged periods of time failed to develop tolerance to either LPS or to HCV core protein, similar to monocytes of HCV-infected patients (Fig 1B and 2B). These data suggested that first, KCs develop tolerance to TLR ligands and second, *in vivo* endotoxemia leads to loss of tolerance to subsequent TLR stimuli in KCs.

Discussion

This study has provided three key observations related to the inflammatory cell activation during cHCV infection. First, we identified that monocytes, the precursors of tissue macrophages, are pre-activated in patients with chronic HCV infection. Second, we demonstrated that *in vivo* occurring concentrations of IFN γ , endotoxin and HCV core protein modulate monocyte functions and favor the increased frequency of MyD88/IRAK complexes, elevated NF κ B activation, and excessive TNF α production seen in cHCV patient's monocytes. Third, our data suggest that the mechanisms by which LPS, HCV core protein and IFN γ amplify the inflammatory monocytes/macrophages activation involve loss of TLR tolerance. Together these data provide evidence for both host and virus-derived factors modulate macrophages for perpetual inflammatory activation during cHCV infection.

Inflammation is a major component of the pathogenesis of cHCV infection (19,22); however its fine mechanisms are yet to be fully understood. TNF α , a potent inducer of inflammation and a key regulator of innate immunity, is beneficial for generation of the initial immune responses against viruses, including HCV (22). However TNF α has multiple harmful effects including induction of hepatocyte apoptosis, aggravation of liver fibrosis, and increase in the frequency of autoimmune diseases associated with HCV (23,24). Increased production of TNF α in T cells, NK, NKT cells and dendritic cells of cHCV-infected hosts was previously reported and may play a detrimental role in HCV clearance (6,22,25). Here we focused our attention on monocytes and tissue macrophages as potential sources for TNF α during cHCV infection to show that cells of patients with chronic HCV infection produce higher amounts of TNF α compared to controls upon stimulation with TNF α -inducing TLR ligands. Under normal regulation TLR tolerance limits TNF α production and it is protective against excessive immune activation and its harmful effects (10). Our data revealed that these protective mechanisms are de-regulated in HCV patients. We provide evidence that HCV patient's monocytes fail to establish homo- and hetero-tolerance to pro-inflammatory cytokine-inducing TLR ligands, including TLR2/TLR1, TLR2/TLR6, TLR4, TLR3 and TLR7/8. Here we showed that HCV core protein, a TLR2 ligand (6,7), activated and induced tolerance in normal but not in cHCV monocytes. We also identified increased levels of endotoxin, a TLR4 ligand, in plasma of patients with cHCV infection, which is in agreement with previous reports (26). It is noteworthy that cHCV patients included in our study did not exhibit liver cirrhosis, yet low levels of endotoxemia occurred in patients with cHCV without advanced liver disease.

IFN γ is pivotal for efficient adaptive immune responses. We identified that IFN γ levels are higher in patients compared to controls, which is in agreement with other reports (27). Further, moderately elevated levels of IFN γ associated with endotoxemia and HCV core protein present in the circulation during cHCV infection may play a detrimental role in the initiation and/or maintenance of the pro-inflammatory activation. *In vitro*, plasma levels of endotoxin comparable to those identified in cHCV patients induced LPS homo-tolerance, while IFN γ could reverse tolerance to TLR ligands. Our data on cHCV monocytes are in agreement with other researchers who observed that *in vivo* IFN γ restored the systemic TNF α response to endotoxin in LPS-desensitized mice and identified that monocytes played a critical role in this process (28). Both beneficial and detrimental effects of IFN γ during bacterial infection have been recognized (29). Our results suggest that moderately elevated levels of IFN γ seen in the

circulation of cHCV patients may favor pro-inflammatory activation in monocytes and, possibly, tissue macrophages. Most importantly, IFN γ is key for establishment of anti-viral immunity and although the levels of IFN γ found in our patients may not be sufficient to support a robust adaptive immune response (22), we show that they are sufficient for pro-inflammatory activation of monocytes/macrophages.

The mechanism of TLR tolerance is complex (30) and it is yet to be fully determined how IFN γ breaks TLR tolerance. Upon stimulation with a specific ligand, most TLRs recruit the common TLR adaptor MyD88, IRAK and TRAF6 proteins and trigger MAPK and NF κ B signaling to produce pro-inflammatory cytokines (4,8). TLR-mediated activation is regulated by expression of TLRs and by inhibitory molecules ST2, SIGIRR, SHIP, TWIST, PI3K, and TTP. We failed to detect differences in protein expression of TLR2, TLR4 or any of the above-mentioned negative regulators in cHCV monocytes compared to controls (data not shown). In TLR ligand-induced tolerance the functions of IRAK1, MAPK, IKK and NF κ B components are impaired (8,9). In contrast, cytokine-induced break in TLR tolerance is characterized by lack of changes in TLR expression, increased formation of MyD88/IRAK complex, increased NF κ B activation and elevated levels of pro-inflammatory cytokine production (8,9,30). We identified that cHCV monocytes exhibited multiple prerequisites of excessive response to TLR ligands: high levels of MyD88/IRAK complexes, pre-activation of NF κ B, and no changes at the levels of TLR proteins or at the level of negative regulators. We observed a similar pattern of changes in TLR-tolerant cells induced *in vitro* by IFN γ treatment, in agreement with other investigators (8,9). These results suggested that the *in vivo* cytokine milieu likely affects the ability of monocytes and tissue macrophages to respond to repeated encounter with TLR ligands during cHCV infection. Our data using *in vitro*-differentiated macrophages support this hypothesis: in contrast to freshly isolated cHCV monocytes, cHCV macrophages differentiated *in vitro* without the cHCV environment developed tolerance to TLR ligands. We also show that the cHCV *in vivo*-conditioning of the monocytes that renders them resistant to TLR-mediated tolerance is reversible upon elimination of the modulatory agents, and the presence of modulatory agents (IFN γ , LPS and HCV core protein) during macrophage differentiation can reproduce the lack of TLR tolerance seen in monocytes.

Based on our results with monocytes/macrophages we, thus, speculate that during cHCV infection HCV core protein may act together with host-derived factors, such as IFN γ and endotoxin, to modulate the function of tissue macrophages, including Kupffer cells. In agreement with other investigators (21), we show that cHCV patients present markers of macrophage activation, such as CD163 and CD33, in the livers, suggestive of *in vivo* KC activation. Using mouse KCs we identified that they are capable of establishing tolerance to TLR ligands in a manner similar to monocytes and macrophages. Further, we show that *in vivo* contact with LPS, a TLR4 ligand, modulates Kupffer cells functions: where short term endotoxemia renders KCs resistant, while prolonged endotoxemia makes KCs sensitive to subsequent TLR-mediated stimulation. Our results using KC's immature myeloid precursors, the monocytes, and their mature forms, the *in vitro*-differentiated human macrophages, as well as mouse KCs lend support to the contention that human KCs in cHCV infection may present a similar pro-inflammatory-activated phenotype.

In conclusion, we propose that IFN γ , endotoxin, and HCV core protein collectively contribute to functional modulation of monocytes and macrophages during cHCV infection. Due to the common cellular origin, it is possible that similar changes may occur in Kupffer cells locally in the liver. Our results suggest novel mechanisms for persistent, immune-mediate inflammation in patients with chronic hepatitis C virus infection.

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Figure 1. Monocytes of cHCV patients fail to develop homo-tolerance to TLR4

Monocytes of controls (n=16) (A) and HCV patients (n=15) (B) were kept in medium or stimulated with TLR4 ligand LPS (100ng/ml) for 24 hrs (1st stimulation) and re-challenged with LPS (100ng/ml) for an additional 24 hrs (2nd stimulation). The production of TNF α in culture supernatants was analyzed in ELISA. Average $\pm$ SD is shown (*p<0.05).

Figure 2. Monocytes of cHCV patients, unlike controls and NASH patients, fail to develop hetero-tolerance to TNF α -inducing TLR ligands

Monocytes from controls (n=16) (A), HCV patients (n=15) (B) and NASH patients (n=6) (C) were cultured in medium or stimulated with LPS (100ng/ml) for 24 hrs (1st stimulation) and re-challenged with LPS (100ng/ml), PGN (5 μ g/ml), Pam₂CSK₄ (100ng/ml), Pam₃CSK₄ (100ng/ml), poly I:C (100ng/ml) or Gardiquimod (5 μ g/ml) for an additional 24 hrs (2nd stimulation). The production of TNF α in culture supernatants was analyzed in ELISA. Average $\pm$ SD is shown (* p<0.05).

Figure 3. cHCV patients exhibit elevated levels of IFN γ , endotoxin, and HCV core protein in the peripheral circulation

(A) The levels of IFN γ in plasma of HCV-infected patients (n=30) and controls (n=18) were quantified using a specific ELISA. The asterisk (*) represents p < 0.05.

(B) The levels of RNA coding for CXCL9, CXCL10, CXCL11 and RIG-I in freshly isolated monocytes were quantified using PCR. Each band represents a pooled sample from 2 controls or 3 cHCV patients.

(C) The levels of endotoxin in plasma of cHCV patients (n=30) and controls (n=18) were quantified using LAL assay; average $\pm$ SE is shown, the asterisk (*) indicates p < 0.05.

(D) The levels of HCV core protein in the plasma of HCV-infected patients (n=30) and control (n=18) were quantified using HCV core ELISA; the asterisk (*) represents $p < 0.05$.

Figure 4. Tolerance to LPS and to HCV core protein is disrupted by IFN γ treatment

(A) Monocytes from controls (n=16) and HCV patients (n=15) were kept in medium or stimulated for 24 hrs (1st stimulation) and re-challenged with HCV core protein for additional 24 hrs (2nd stimulation). The production of TNF α was analyzed in culture supernatants using ELISA and the * indicates p<0.05 (panels A–D).

(B) Monocytes of controls (n=3) were stimulated with LPS (100pg/ml) for 24 hrs (1st stimulation) and re-challenged with LPS for 24 hrs (2nd stimulation). IFN γ (100pg/ml) was added 4 hrs before and during the 1st stimulation.

(C) Monocytes of controls (n=3) were stimulated with HCV core (10000fMol/L) for 24 hrs (1st stimulation) and re-challenged with HCV core (25000fMol/L), for 24 hrs (2nd stimulation). IFN γ (100pg/ml) was added for 4 hrs before and during the 1st stimulation.

(D) Monocytes of controls (n=3) were stimulated with LPS (100pg/ml) for 24 hrs (1st stimulation) and re-challenged with HCV core (25000 fMol/L) for 24 hrs (2nd stimulation). IFN γ (100pg/ml) was added for 4 hrs before and during the 1st stimulation.

Figure 5. HCV-infected patients' monocytes show elevated NFκB activity and increased frequency of MyD88/IRAK complexes, similar to IFN γ +LPS-stimulated normal monocytes

(A) Normal monocytes were pre-treated *in vitro* with IFN γ followed by stimulation with LPS for 1 hour, as indicated. Five μ g of nuclear protein were analyzed for NFκB binding capacity in EMSA using a radioactive labeled NFκB-specific oligonucleotide. A cold NFκB oligonucleotide incubated with nuclear protein from LPS-stimulated sample was used to determine the specificity of NFκB band (Comp). A representative EMSA gel is shown on the top and densitometric analysis of n=4 is shown on the bottom.

(B) The NFκB binding capacity of normal and cHCV monocytes was analyzed in EMSA, as described above. Each band represents 5 μ g of nuclear proteins from a pool of 2 controls or 3

HCV patients. Normal monocytes (corresponding to the pool #2) were stimulated with LPS (100ng/ml for 1hr *in vitro*) and used as positive control for this assay (Normal+LPS). A cold NFκB oligonucleotide, incubated with nuclear protein from LPS-stimulated sample was used to determine the specificity of NFκB band (Comp). The densitometric analysis of the EMSA gel is shown on the top as average±SD and a representative gel is shown at the bottom.

(C) Normal monocytes were pre-treated *in vitro* with IFNγ followed by stimulation with LPS for 1 hour. Five hundred μg of protein were incubated with anti-MyD88 antibody at +4°C overnight; the loading of equal protein amounts included in the immunoprecipitation assay was conformed by immunoblotting against β-actin (input). The amount of IRAK1 immunoprecipitated with anti-MyD88 antibodies was detected by western blot (bottom band) and quantified by densitometric analysis; shown is average±SD from n=3.

(D) Equal amounts (500 μg) of total protein from freshly isolated monocytes of controls (n=10) and cHCV patients (n=15) were incubated with anti-MyD88 antibody at +4°C overnight; each band represents a pool of 2 controls or 3 HCV patients. The amount of IRAK1 immunoprecipitated with anti-MyD88 antibodies was detected and quantified as above; shown is average±SD.

Figure 6. IFN γ is capable of breaking TLR tolerance in macrophages

(A) Macrophages of controls (open bars) and cHCV patients (black bars) were differentiated *in vitro*, then stimulated with LPS (10pg/ml) for 24 hrs (1st stimulation) and re-challenged with LPS (100pg/ml), for additional 24 hrs (2nd stimulation). Where indicated IFN γ (100pg/ml) was added during the differentiation and 1st stimulation. The production of TNF α in the culture supernatants was analyzed in ELISA from n=6 (A–C).

(B) Macrophages were differentiated as above, then stimulated *in vitro* with LPS (10pg/ml) for 24 hrs (1st stimulation) and re-challenged with HCV core (25000 fMol/L) for additional 24 hrs (2nd stimulation).

(C) Macrophages were differentiated *as above*, then stimulated with HCV core (10000 fMol/L) for 24 hrs (1st stimulation) and re-challenged with HCV core (25000 fMol/L), for additional 24 hrs (2nd stimulation).

Figure 7. The patients with cHCV infection exhibit elevated levels of TNF α , CD163 and CD33 in livers

The levels of RNA coding for TNF α (A), CD163 (B) and CD33 (C) were analyzed in livers of controls (n=3) and HCV patients (n=12) using real-time quantitative PCR. The asterisk (*) represents $p < 0.05$.

(D) The mice were injected i/p with saline, LPS (5mg/kg) one time (the “1 dose of LPS” group) or 5 times (the “5 dose of LPS” group received 1mg/kg LPS every 3 days, total of 5 doses) and the Kupffer cells were isolated. *In vitro* the cells were stimulated with LPS (100ng/ml) or HCV core protein (25000 fMol/L) and the TNF α in culture supernatants was quantified using specific

ELISA. The asterisk (*) represents $p < 0.05$ using t-test from $n=4$ in saline and $n=3$ in LPS-treated groups.

Table 1

The characteristics of patients included in the study.

Parameter	HCV patients (Value)	NASH patients (Value)
Age (years)	46±9	45±8
Gender: Male	22	2
Female	8	4
AST (U/l)	72±35	62±28
ALT (U/l)	84±41	76±31
Viral load	$1.92 \times 10^6 \pm 0.3 \times 10^6$	0
Viral genotype 1a	21	Not detected
1b	3	Not detected
2b	2	Not detected
3a	1	Not detected
undetermined	3	Not detected
Liver biopsy performed	26	6
Liver fibrosis	6	0
Ishak Stage 1–2	5	0
Ishak Stage 3–4	1	0
Liver inflammation	26	6
Mild (Ishak Score 0–6)	11	5
Moderate (Ishak Score 7–12)	14	1

Table 2

PCR primers.

Target	Primer	Sequence 5'- 3'
CD163	forward	AGCTCTCTGGGACTGCACAT
	reverse	ACACACAGTCCCCCACTCTC
CD33	forward	TGTTCCACAGAACCCAACAA
	reverse	TTCCTCCTGTGGGTCTTCAC
CXCL-9	forward	AAAAGTGGGAGAAACAGGTCAGC
	reverse	TTGTGAGTGGGATGTGGTTGG
CXCL-10	forward	AGTGGCATTCAAGGAGTACCT
	reverse	ATCCTTGGAAGCACTGCATCG
CXCL-11	forward	AAGTAGCAGCAACAGCACCAGC
	reverse	GGTCCTTTCACCCACCTTTCATC
RIG-I	forward	CCAGATGCCAGACAAAGATGAAGAG
	reverse	GCTCGGACATTGCTGAAGAAGTC
18S	forward+reverse	Ambion (Austin, TX)
TNF α	forward+reverse	MaximBio (San Francisco, CA)