

Review

Modulation of monocyte/macrophage-derived cytokine and chemokine expression profile by persistent Hepatitis C virus (HCV) infection leads to chronic inflammation

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Received on November 21, 2011; Accepted on January 13, 2012; Published on February 15, 2012

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Abstract

HCV infection presents a major public health problem, with more than 170 million people infected worldwide. Chronicity and persistence of infection constitute the hallmark of the disease. Although HCV is a hepatotropic virus, subsets of immune cells have been found to be permissive to infection and viral replication. Peripheral blood monocytes, attracted to the site of infection and differentiated into macrophages, and resident hepatic macrophages, known as Kupffer cells, are im-

portant mediators of innate immunity, through production of several chemokines and cytokines in addition to their phagocytic activity. HCV proteins have been shown to modulate the cytokine and chemokine production profile of monocytes/macrophages, as it is suggested by both *in vitro* and clinical studies. This modified expression profile appears crucial for the establishment of aberrant inflammation that leads to liver cirrhosis and hepatocellular carcinoma.

Introduction

HCV is a single-stranded, positive-sense RNA virus of the Flaviviridae family. Naturally occurring variants are classified into 6 major genotypes, each presenting with many subtypes (Bostan & Mahmood 2010). According to the World Health Organization approximately 170 million people are chronically infected with HCV and it is estimated that about 3–4 million new infections occur each year. HCV establishes persistent infection in 80% of the cases, leading to liver cirrhosis and/or hepatocellular carcinoma (HCC). As a result, more than 350,000 deaths occur due to HCV-related liver diseases per year. To date, the molecular mechanisms used by the virus to establish chronicity are under intense investigation.

Briefly, the HCV life cycle consists of binding and entry of the virion, cytoplasmic release and uncoating of the viral particle, internal ribosome entry site (IRES)-mediated translation of the viral genome and polyprotein processing, RNA replication, packaging and assembly and finally virion maturation and release (Moradpour *et al.* 2007). The mechanism of viral entry has been studied in depth. Infection of a host cell depends on the interactions between the E1 and E2 glycoproteins of the HCV particle and several

cell-surface receptors and proteins. The most essential receptors for viral binding and entry are CD81 and scavenger receptor class B, type I (SR-BI). The tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) are needed as cofactors (von Hahn & Rice 2008). In addition, recent studies suggest that low density lipoprotein receptor (LDLR) (Popescu & Dubuisson 2010) plays a pivotal role in HCV infection (Figure 1). Upon viral entry, translation of the viral RNA is directed by a highly organized IRES found within the 5' UTR. The single polyprotein produced, is further processed in three structural (core, E1, E2), p7 and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) by host peptidases and viral proteases (Dubuisson 2007). A new viral protein encoded by an alternative reading frame within the core coding region, named alternative reading frame protein (ARFP), F, or core+1 protein, was discovered independently by different laboratories, including ours (Figure 2). It has been suggested that it may be responsible for some of the host-virus interactions attributed to core (Varaklioti *et al.* 2002, Walewski *et al.* 2001, Xu *et al.* 2001). Viral replication occurs in the host cytoplasm through double-stranded RNA intermediates bound to modified areas of the ER-membranous compartments (Chisari 2005). Following replication,

the particle assembly happens on lipid droplets, where HCV particles are attached to or incorporated into very low-density lipoproteins (VLDL) during assembly of lipoprotein particles and secreted together with VLDL (Miyanari *et al.* 2007). Thus, it is believed that the virus creates lipid-rich cellular surroundings in order to complete its own life cycle. In addition, virion-lipid association in serum circulation may help the virus escape from immune surveillance through masking of putative viral antigenic moieties (Syed *et al.* 2010).

Viral clearance, during the acute phase of the infection, requires strong IFN- γ +, HCV-specific CD8+ and T_H1 CD4+ T-cell responses (Elliot *et al.* 2006, Koziel 2005). However, in most infected patients HCV is able to disrupt both innate and adaptive immune responses, by modulating the expression and function of all cells of the immune system, including monocytes and macrophages (Caussin-Schwemling *et al.* 2001, Szabo *et al.* 2007, Thimme *et al.* 2011). This leads to persistent infection and chronic inflammation. This review will focus on the interplay of HCV with monocytes/macrophages and the altered cytokine and chemokine profile, typical of the aberrant inflammation that characterizes persistent HCV infection.

HCV lymphotropism

Liver is the target organ of HCV. However, several other types of cells/tissues have been tested for viral infection and replication, like biliary cells, salivary gland, pancreatic epithelium, thyroid, bone marrow, adrenal gland, spleen, lymph nodes and brain. One of

the most interesting and well-studied potential extra-hepatic viral reservoirs may be the cells of the immune system (Zignego *et al.* 2007). So far, there has been sufficient evidence of viral replication in almost all the subsets of immune cells, such as granulocytes, monocytes/macrophages, dendritic cells (DCs), B- and T-lymphocytes. Several *in vivo* and *in vitro* studies have shown the presence of both positive and negative RNA strands in subsets of immune cells, a strong indication of viral replication (Bare 2009, Revie & Salahuddin 2011).

The presence of HCV-specific receptors and entry cofactors on the surface of immune cells enhances the possibility for viral entry. However, a recent study by Marukian and coworkers (Marukian *et al.* 2008) suggested that SR-BI and CLDN1 have either undetectable or very low levels of expression in B-, T- and natural killer (NK) cells; therefore, they may not support viral entry. On the other hand, new evidence implicates LDLr (Popescu & Dubuisson 2010) and Fc γ R2 (Revie & Salahuddin 2011) in viral entry. Both of these receptors are present in most subsets of immune cells. Finally, Natarajan *et al.* (2010) suggest that HCV is carried predominantly on the surface of peripheral blood mononuclear cells (PBMCs), which allows the virus to spread to distant sites, such as lymph nodes, spleen, kidneys and the central nervous system (CNS). Extra-hepatic HCV transport and lymphotropism could account for several lymphoproliferative and autoimmune disorders observed in patients with chronic Hepatitis C (CHC) infection, including cutaneous vasculitis, glomerulonephritis, neuropathy,

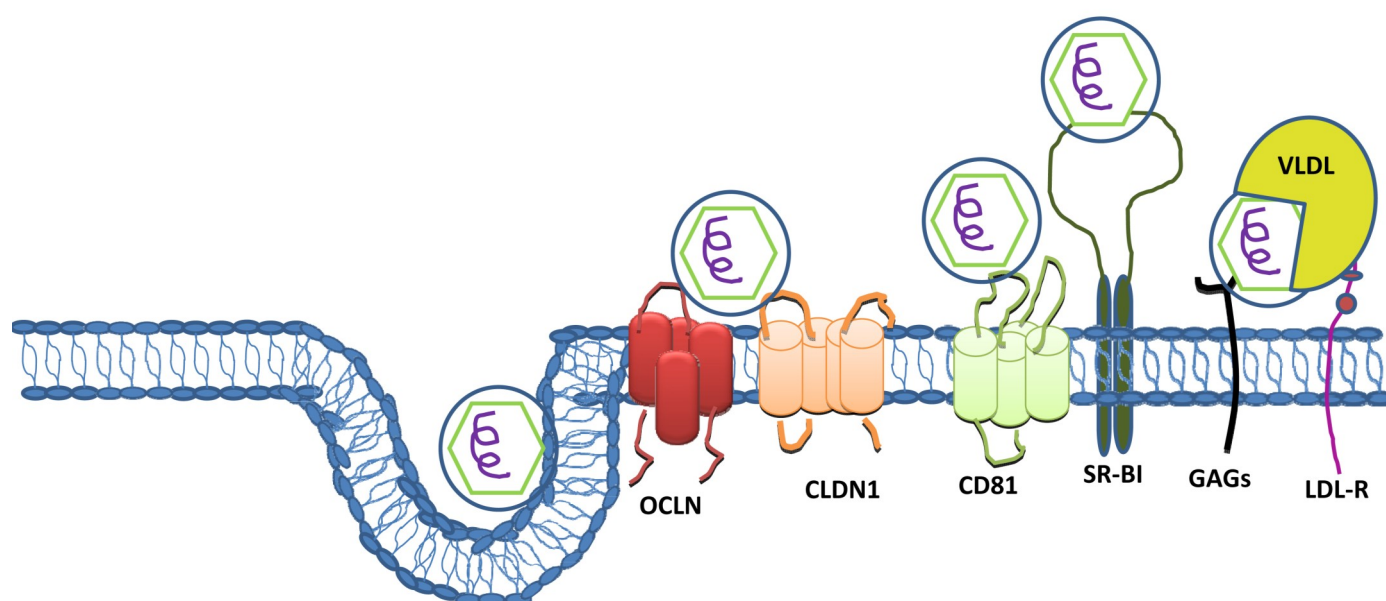


Figure 1. HCV entry. Virion's E1 and E2 glycoproteins interact with several cell-surface (co-)receptors and proteins. The essential receptors for viral binding and entry are CD81, scavenger receptor class B, type I (SR-BI), the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) and low density lipoprotein receptor (LDLr).

non-Hodgkin lymphoma, mixed cryoglobulinemia and CNS disorders (Charles & Dustin 2009, Racanelli & Rehmann 2003). In addition, the existence of a long-term reservoir that may help the virus establish persistence may be advantageous to the virus. Such a reservoir may account for viral recurrence after liver transplantation or viral clearance, chronic immune dysfunction and resistance to anti-viral treatment (Bare 2009).

Still, a lot of debate exists concerning both the specificity and significance of HCV lymphotropism. The hypotheses that argue against extra-hepatic replication of the virus and the existence of a long-term viral reservoir in immune cells, are based on studies showing that either HCV RNA could not be detected in PBMCs or the percentage of infected PBMCs was very low to be of importance in the subjects tested, therefore the virus was considered cleared (Bare 2009). However, these discrepancies can be explained in part by a genotype-dependent lymphotropism of the virus. For example, although results of an *in vitro* study in which JFH-1 strain (genotype 2a) provided no evidence of infection in monocytes, B-, T- cells and DCs, others found that patients infected with HCV genotype 1 (mainly 1b) demonstrated higher detection rates of HCV in their PBMCs (Zignego *et al.* 2007). Finally, another factor that may play a role in HCV lymphotropism may be the presence of HCV variants in patient serum. In patients tested for HCV genomic variant composition, a selective compartmentalization of distinctively different variants was observed between liver, serum and lymphoid cells. A possible explanation for certain variants being selectively lymphotropic over others, may be that certain tissue-specific factors favor the selection of one virus variant over another (Bare 2009, Revie & Salahuddin 2011, Zignego *et al.* 2007).

Monocytes and Macrophages in HCV Infection

Macrophages are the main terminally differentiated cells of the mononuclear phagocyte system and key cells of the innate immune response. Macrophages originate in the bone marrow, which contains all macrophage precursor cells. Newly formed monocytes leave bone marrow and circulate in peripheral blood,

where they normally make up 1-6% of the total PBMCs. They migrate into extravascular tissues, adhere to the endothelium through interactions of surface glycoproteins like LFA-1 (lymphocyte function-associated antigen 1) with ICAM-1 (intercellular adhesion molecule-1), and differentiate into resident tissue macrophages. Depending on the tissue of residence, macrophages of the liver are called Kupffer cells, lung resident macrophages are called alveolar macrophages, whereas macrophages of the CNS, bone, skin and connective tissue are named microglia, osteoclasts, Langerhans' cells and histiocytes, respectively (Lewis 1992). Macrophages remain in tissues for several months and have a number of important functions. They maintain tissue homeostasis by phagocytosing apoptotic cells and producing growth factors, such as macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), which stimulate the production and function of mononuclear cells. Additionally, they recognize foreign antigens through a broad range of surface pathogen recognition receptors and present those to T-helper (T_H) cells through the major histocompatibility complex II (MHC-II) (Geissmann *et al.* 2010).

One of the most important functions of macrophages is the production of cytokines and chemokines. Cytokines are small "hormone-like" proteins that act as chemical messengers between various cells in a paracrine or autocrine manner. They affect nearly every biological process, from response to infection and antigens to embryonic development, cell differentiation, protection against tumor cells, changes in cognitive functions and progression of the degenerative processes of aging (Dinarelo 2007). Heparin-binding cytokines that direct the recruitment of immune cells to inflammatory sites are called chemokines. (Heydtmann & Adams 2009, Kang & Shin 2011). Cytokines are generally classified as pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines, like interleukin-1 and -6 (IL-1 and IL-6), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) are present at high levels in chronic disease, such as cancer and auto-immune diseases. In such cases, overproduction of these otherwise "beneficial" molecules may result in aberrant inflammation, persistent T-cell acti-

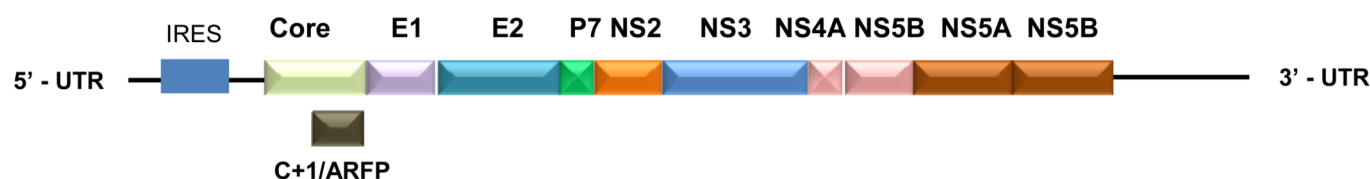


Figure 2. Schematic representation of HCV genome organization and polyprotein. HCV encodes a single polyprotein with the structural proteins and the nonstructural proteins, which are further processed by viral and host signal peptidases. 5'UTR, IRES and 3'UTR are also depicted.

vation and exacerbation of disease pathology. On the other hand, anti-inflammatory cytokines are immunomodulatory molecules that control the activity of inflammatory reactions. Cytokines in this group include interleukins IL-4, IL-10, IL-11 and IL-13 and interleukin 1-receptor α (IL-1Ra). Notably, only IL-1Ra and IL-10 are produced by monocytes/macrophages (Akdis *et al.* 2011, Dinarello 2007, Opal & DePalo 2000).

HCV has been shown to interact with monocytes and macrophages affecting many of their functions. Upon HCV infection, the activation of the host monocytes/macrophages is essential in initiating an inflammatory response. It is triggered by the recognition of viral patterns mediated through specialized membrane and intracellular receptors, leading to the production of cytokines and chemokines. However, HCV has the ability to direct this reaction in favor of its own replication, establishing a persistent infection (Szabo *et al.* 2007). HCV interacts with monocytes and macrophages directly or indirectly, thus altering their cytokine/chemokine profile and impairing both the innate immune response and the priming of adaptive immune response.

HCV elicits monocyte/macrophage-mediated modulation of cytokine and chemokine expression profile

HCV exerts a direct action on monocytes/macrophages either by infecting them or through the interaction of cellular receptors with extracellular viral proteins. As a result, it affects the expression profile of circulating pro-, anti-inflammatory cytokines and chemokines. In addition, many of these have been shown to be differentially regulated in liver biopsies from HCV infected patients (Antonelli *et al.* 2009, Chen *et al.* 2007, Larubia *et al.* 2008, Migita *et al.* 2006, Nischalke *et al.* 2004, Spanakis *et al.* 2002, Zekry *et al.* 2002, Zeremski *et al.* 2007).

Most of the regulatory effects of HCV viral proteins on cytokine and chemokine gene expression occur through the Toll-like receptor pathway (TLR). TLRs are pathogen recognition receptors. They act as signal transducers leading to HCV-mediated modulation of the cytokine/chemokine expression profile. For example, HCV proteins NS3, NS2, NS4A and NS5A have been suggested to disrupt TLR signaling (Abe *et al.* 2007). Besides signal transduction, TLRs have been known to be expressed in monocytes derived from HCV infected patients, where TLRs 2, 3, 5, 6, 7, 8, 9, and 10 showed higher mRNA expression levels compared to monocytes from healthy individuals (Dolganiuc *et al.* 2006b). Furthermore, such mono-

cytes have lost TLR tolerance, thus they respond to consecutive stimuli, like core protein, producing enormous amounts of cytokines. This loss of TLR tolerance has also been shown in Kupffer cells from HCV patients (Dolganiuc *et al.* 2007).

Cytokines in HCV infection

Interleukin-6

IL-6 is a multifunctional cytokine produced by endothelial cells, fibroblasts, monocytes/macrophages and hepatocytes. IL-6 is an effector of both innate and adaptive immunity by enabling terminal differentiation of B-cells, eliciting hepatic acute phase response, differentiating and/or activating macrophages and T-cells and rapidly inducing peripheral blood neutrophils (Akira & Kishimoto 1992). HCV up-regulated IL-6 expression in *ex vivo* monocytes and macrophages isolated from HCV-infected patients and healthy donors, in both mRNA and protein levels. This effect was exerted mainly by extracellular core protein and to a lesser extent by extracellular NS3 (Feldmann *et al.* 2006). However, while Fiorucci and collaborators studied the effect of core and ARFP/core+1 in monocytes/macrophages in parallel, they ended up attributing this function to the newly discovered ARFP/core+1 protein (Fiorucci *et al.* 2007). On the other hand, Chung *et al.*, in two different studies (2010, 2011), suggested that monocytes isolated from HCV-infected patients showed reduced IL-6 production when treated primarily with extracellular core and subsequently with TLR 2 and 4 ligands (Homo- and Cross-Tolerance). IL-6 up-regulation by core and NS3 was performed through a TLR-signaling pathway, leading to the suggestion that TLR-2 is the major receptor and TLR-1/6 are the co-receptors (Chang *et al.* 2007). Subsequently, IL-6 acted in an autocrine or paracrine manner activating the Janus Kinase/Signal Transducer and Activator of Transcription (Jak/STAT) pathway through phosphorylation of STAT3 (Tacke *et al.* 2011).

Tumor necrosis factor α

Tumor necrosis factor α (TNF- α) belongs to the TNF superfamily, which also contains TNF- β . It is predominantly produced by activated monocytes and macrophages and to a lesser extent by other cell types, such as NK-cells and hepatocytes. TNF- α is a multifunctional cytokine that induces inflammation, acute phase response and apoptosis, activates antigen presentation and up-regulates adhesion molecules and several chemokines (Aggarwal 2003). It has been shown that incubation of human macrophages with positive HCV sera of genotypes 1b and 3a resulted in TNF- α up-

regulation (Radkowski *et al.* 2004). Up-regulation of TNF- α is attributed mainly to core and NS3, although NS5A was also shown to be implicated in the process (Hosomura *et al.* 2011). The up-regulation was TLR-dependent, with TLR-2 being the essential receptor and TLR-1/-6, and possibly others, acting as co-receptors (Chang *et al.* 2007, Dolganiuc *et al.* 2004). There is corroborating evidence regarding the effect of the virus on monocyte-derived TNF- α is concerned. In one study, infection of monocytes with JFH-1 strain resulted in down-regulation of TNF- α after a week of culture (Eksioglu *et al.* 2010). In another study increased TNF- α levels were observed in monocytes isolated from patients with chronic HCV infection who had received no antiviral treatment. TNF- α up-regulation resulted in core-mediated reduction of IFN- α expression by plasmacytoid dendritic cells (PDCs) after stimulation with TLR9 ligands (Dolganiuc *et al.* 2003, 2006a). In general, TNF- α balanced production seems to be important for viral clearance, as monocytes from patients with self-limited HCV infection produce less TNF- α after stimulation with core in comparison to monocytes from chronic HCV patients (Martin-Blondel *et al.* 2009).

Interleukin-1

IL-1 β accounts for 90% and *IL-1 α* for 10% of IL-1 production from monocytes upon stimulation. IL-1 is a strong inducer of other pro-inflammatory cytokines, such as TNF- α and IL-6. It is involved in hematopoiesis and the differentiation of certain subsets of T-cells (Akdis *et al.* 2011). IL-1 has been suggested to inhibit HCV RNA replication in a subgenomic replicon cell line and IL-1 β protein levels were found increased in the serum of HCV patients. Based on the above, IL-1 presents an important cytokine for HCV elimination however, very little is known about the way that this cytokine is affected by the virus (Zhu & Liu 2003). In a study by Hosomura N. and coworkers, (Hosomura *et al.* 2011), where the effect of HCV proteins (core, NS3, NS4, NS5) on Kupffer cells isolated from healthy donors was examined, a significant up-regulation of IL-1 β caused by all HCV proteins except core was observed. On the other hand, NS3 and NS4A have been found to decrease IL-1 α production, when stably expressed in mouse macrophage cell lines (Abe *et al.* 2007). Finally, it has been proposed that an imbalance between IL-1 β and its antagonist IL-1R α , may contribute to the pathogenesis of HCV (Gramantieri *et al.* 1999).

Interleukin-12

IL-12 is a pro-inflammatory cytokine, expressed mainly by phagocytes. It is responsible for the induc-

tion of T_{H1} differentiation and cytotoxicity and for the increased NK-cell cytotoxicity (Akdis *et al.* 2011). HCV has been suggested to down-regulate the production of IL-12 by monocytes/macrophages, impairing the priming of adaptive immune responses. This effect was exerted by core protein and it has been studied either by adding extracellular protein or by infection of monocytes with HCV JFH-1 (Eisen-Vandervelde *et al.* 2004, Eksioglu *et al.* 2010, Waggoner *et al.* 2005). The inhibition of IL-12 by core was linked to the up-regulation of programmed death-1 (PD-1) receptor. Binding of PD-1 to its ligand (PDL-1/PDL-2) led to a cascade that transmitted inhibitory signals to downstream signaling pathways (Zhang *et al.* 2011a). PD-1 was found to be up-regulated in monocytes isolated from chronic HCV patients. PD-1 up-regulation was attributed to ligation of core to the receptor of C1q complement (gC1qR) and it was reversed upon disruption of this interaction, leading to restored IL-12 levels (Eisen-Vandervelde *et al.* 2004, Waggoner *et al.* 2005). In addition, up-regulation of two other key molecules, T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) and suppressor of cytokine signaling-1 (SOCS-1), was part of the mechanism responsible for decreased IL-12 production, through crosstalk with PD-1 (Zhang *et al.* 2011a, b). Furthermore, it has been suggested that PD-1 acts via inhibition of STAT1 phosphorylation and that the phosphoinositide 3-kinase (PI3-K) pathway is necessary for the impairment of IL-12 production (Ma *et al.* 2011). Apart from PD-1, PDL-1 (B7-H1) was also shown to be up-regulated in monocytes isolated from patients with chronic HCV infection. This increase led to impaired proliferation of HCV specific T-cells, through a PD-1/PDL-1 interaction (Jeong *et al.* 2008). Blockade of this interaction in an HCV core murine model resulted in restored T-cell functions (Lukens *et al.* 2008). Finally, Brady *et al.* and Sene *et al.*, demonstrated that NS4 and NS5A proteins, respectively, were responsible for the impaired IL-12 production by monocytes and that NS5A-mediated regulation of IL-12 was through TLR-4 (Brady *et al.* 2003, Sene *et al.* 2010).

Interleukin-10

IL-10 is a major anti-inflammatory cytokine produced by B-cells, T-cells, monocytes/macrophages and DCs. It functions as an immune suppressor. Therefore, down-regulation of IL-10 production may lead to impaired immune manifestations or to persistent inflammation (Akdis *et al.* 2011). IL-10 is up-regulated by HCV and almost all viral proteins have been shown to modulate its expression. Infection of monocytes with JFH-1 strain resulted in up-regulation of IL-10 (Eksioglu *et al.* 2010). Treatment of monocytes and macrophages

with extracellular core or NS3 resulted in a TLR-2-mediated IL-10 induction with TLR-1/6 acting as co-receptors (Chang *et al.* 2007). Similar results were obtained following treatment of Kupffer cells with several HCV proteins. IL-10 up-regulation was achieved by NS3, NS4 and NS5 proteins, but not core (Hosomura *et al.* 2011). In addition, monocytes isolated from both healthy donors and HCV infected patients and treated with NS4, showed increased IL-10 production (Brady *et al.* 2003). Moreover, NS5A appeared to induce IL-10 expression in monocytes through a TLR-4 pathway. Consequently, TLR4 promoted p38- and PI3-K-mediated production of IL-10 (Sene *et al.* 2010). Several immune dysfunctions have been attributed to this altered IL-10 expression profile by monocytes/macrophages, such as impaired IFN- α production by PDCs (Dolganiuc *et al.* 2006a), inhibition of HCV-specific T_{H1} and T_{H17} (IL-17 producing T-cells) responses (Rowan *et al.* 2008) and impaired NK-cells function through a TGF- β -dependent down-regulation of NKG2D receptor. Finally, increased production of IL-10 by monocytes has been linked to chronic infection (Sene *et al.* 2010). Conversely, a more balanced IL-10 production leads to self-limited infection and viral clearance (Martin-Blondel *et al.* 2009).

Transforming growth factor- β

Transforming growth factor β (TGF- β) is an important cytokine/growth factor of innate immune response. It is secreted by monocytes, macrophages, T-cells and other cell types. TGF- β mainly exerts immunosuppression functions, as it inhibits macrophages, T-cell proliferation and functions and B-cell proliferation (Wahl *et al.* 1990). Apart from its immunosuppressive function, TGF- β stimulates accumulation and proliferation of fibroblasts and deposition of extracellular matrix (Lee & Friedman 2011). In addition, it can induce cell transformation and is involved in tumor generation and progression (Akhurst & Derynck 2001). HCV has been found to enhance TGF- β production through HCV NS4 (Rowan *et al.* 2008) and NS5A (Sene *et al.* 2010) proteins. This TGF- β secretion profile enables viral persistence as it leads to impaired T-cell and NK-cell function.

Chemokines in HCV infection

The major chemokines studied in HCV are monocyte chemotactic protein-1 (MCP-1) or CCL2, macrophage inflammatory protein 1 α (MIP-1 α) or CCL3, macrophage inflammatory protein 1 β (CCL4, MIP-1 β), regulated upon activation, normal T-cell expressed and secreted (CCL5, RANTES), interleukin-8 (IL-8,

CXCL8) and interferon γ -inducible protein 10 (CXCL10, IP-10) (Wald *et al.* 2007). All the above mentioned chemokine levels were increased in both sera and liver tissue of patients with chronic HCV infection. Monocytes and macrophages express chemokines either in basal levels or upon stimulation. Despite their importance, there are only a few studies examining the effect of HCV on their production by monocytes or macrophages.

Monocyte chemotactic protein-1

MCP-1 functions as a recruiting factor for monocytes into foci of active inflammation (Deshmane *et al.* 2009). Until today, only ARFP/core+1 protein of HCV has been found to up-regulate the expression of MCP-1 in THP-1 monocytic cell line but not human monocyte-derived macrophages (HMDMs) (Fiorucci *et al.* 2007).

Macrophage inflammatory protein-1

MIP-1 α and MIP-1 β also act as chemotactic factors (Menten *et al.* 2002). Monocytes of HCV patients seem to secrete higher amounts of MIP-1 β and MIP-1 α in comparison to monocytes of healthy individuals (Perrin-Cocon *et al.* 2008). Furthermore, MIP-1 β has been suggested to be up-regulated by ARFP/core+1 protein in both THP-1 cells and HMDMs (Fiorucci *et al.* 2007).

Interleukin-8

IL-8 is produced by many cell types like immune cells, endothelial and epithelial cells, fibroblasts and hepatocytes. Its major functions are chemoattraction of several immune cell subsets, mobilization of hematopoietic stem cells and angiogenesis (Akdis *et al.* 2011). The effect of HCV on IL-8 production by monocytes has been studied extensively. IL-8 is up-regulated in HCV infection. HCV patients' monocytes appear to secrete higher amounts of IL-8 in comparison to those of healthy donors. Moreover, macrophages of cirrhotic patients showed significant IL-8 up-regulation compared to non-cirrhotic and healthy controls (Zimmermann *et al.* 2011). Treatment of primary macrophages with HCV infected patient sera led to significant up-regulation of IL-8 production (Radkowski *et al.* 2004). Furthermore, HCV core has been suggested to up-regulate IL-8 in both monocytes isolated from HCV patients (Feldmann *et al.* 2006) and THP-1 monocytic cells (Fiorucci *et al.* 2007), through a TLR-2 mediated pathway.

Chemokine receptors

IL-8 receptor (CXCR1) was found more highly ex-

pressed in monocytes of cirrhotic patients than non-cirrhotic and healthy controls. These monocytes/macrophages were polarized towards the alternatively activated M2 macrophage phenotype, which plays an important role in hepatic fibrosis (Zimmermann *et al.* 2011). Another chemokine receptor, *CCR7*, showed differential expression between self-limiting infection patients and those with chronic infection or sustained virological response. Infected patients who managed to clear the virus without receiving treatment, had higher levels of *CCR7*, suggesting a possible involvement of this receptor in virus elimination (Martin-Blondel *et al.* 2009). Finally, reduced cell surface *CCR5* has been found in monocytes treated with HCV E2 protein, which resulted in intracellular accumulation of this receptor (Nattermann *et al.* 2004).

Pathogen-derived chemoattractant peptides

Several pathogen peptides, such as HIV-derived peptides T-20 and T-21 and bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) act as chemoattractants, upon ligation at formyl peptide receptor (FPR), a classic chemotactic receptor. Recently, an HCV-derived peptide named C5A has been suggested to function as a potent chemoattractant for monocytes and neutrophils. C5A is a proteolytically cleaved product of NS5A by caspase 3 and 6 and by Ca^{2+} -dependent calpain proteases and corresponds to the membrane anchor domain of NS5A protein. Upon ligation to FPR, C5A promotes up-regulation of monocytic CD11b expression, a subunit of CD18 integrin responsible for adhesion, migration, chemotaxis, phagocytosis, and further degranulation of neutrophils along with increased production of reactive oxygen species (ROS; Lin *et al.* 2011).

Preliminary results from our laboratory, using a silent HSV-1 (Herpes Simplex Virus-1) amplicon-based vector to express HCV core/core+1 viral proteins in HMDMs, showed that core+1 mediated a rise in MIP-1 β gene expression, in addition to a strong up-regulation of IL-10 by both proteins (Kochlios *et al.* 2010).

Cytokine and chemokine profile of monocytes/macrophages is altered via indirect interactions of HCV with other target cells

Within the liver's basic anatomical unit, the sinusoid, hepatocytes secrete several cytokines and chemokines in order to communicate with neighboring cells, such as stellate cells and resident macrophages, as well as PBMCs and DCs of the blood circulating into the sinusoid. HCV or specific viral proteins have been sug-

gested to alter the expression profile of many pro-inflammatory cytokines and chemokines produced by hepatocytes, thereby affecting their expression by adjacent monocytes/macrophages in a paracrine manner. For example, IL-6 up-regulation has been suggested to be mediated by core and NS5A proteins (Ait-Goughoulte *et al.* 2010, Basu *et al.* 2006, Li *et al.* 2011, Macdonald *et al.* 2003), whereas TNF- α was found up-regulated by NS3 (Li *et al.* 2011). In addition, increased IL-32 mRNA levels have been found in hepatocytes of chronic HCV patients (Moschen *et al.* 2011). As far as chemokines are concerned, core induced increased hepatocytic levels of IL-8 (Clement *et al.* 2010, Green *et al.* 2006), MIP-1 β (Clement *et al.* 2010, Harvey *et al.* 2003, Li *et al.* 2011), MIP-1 α (Li *et al.* 2011), MCP-1 (Soo *et al.* 2002), IP-10 (Harvey *et al.* 2003, Jouan *et al.* 2011, Li *et al.* 2011, Zeremski *et al.* 2008) and RANTES (Harvey *et al.* 2003, Jouan *et al.* 2011, Li *et al.* 2011, Soo *et al.* 2002). Results from our laboratory confirm the HCV effect on cytokines and chemokines by core protein (MCP-1, IL-10, TNF- α , IL-6), attributing some of these properties to the newly discovered ARFP/core+1 protein (IL-10, TNF- α) (Kochlios *et al.*, 2010). Upon infection, hepatocytes produce pro-inflammatory cytokines and present viral antigens through the MHC-I pathway, in order to be recognized by monocytes/macrophages and induce phagocytosis. It has been proposed that HCV may, in fact, affect the MHC-I presentation pathway in hepatocytes (Zimmermann *et al.* 2008). Since infected hepatocytes constitute the first line of defense against viral infection, both modulation of the cytokine profile and deregulation of the MHC-I complex leads to great impairment of the innate immune responses.

NK-cells are large, granular lymphocytes, important for host defense against tumors and cells infected with viruses. Upon recognition of a target cell, NK-cells secrete IFN- γ and TNF- α in addition to their "cell killing" capacity (Kindt *et al.* 2006). Cytokine secretion seems to be important for HCV clearance, as it has been suggested that IFN- γ inhibits protein synthesis and RNA replication of subgenomic and genomic HCV replicons (Frese *et al.* 2002) and JFH-1 infection and replication in human hepatocytes (Wang *et al.* 2008). Although NK-cells of chronic HCV patients show an unimpaired cytotoxic activity, their cytokine secretion profile is severely affected, leading to a decreased production of IFN- γ (Ahlenstiel *et al.* 2010, Dessouki *et al.* 2010). HCV E2 glycoprotein was shown to interact with CD81 receptor on the NK-cell surface, thereby modulating Erk phosphorylation and inhibition of IFN- γ production (Bowen & Walker 2005, Crotta *et al.* 2002, Szabo *et al.* 2007). Furthermore, NK-cells of HCV patients demonstrated in-

creased IL-10, TGF- β and IL-8 production, which may facilitate HCV persistence (Cheent & Khakoo 2011). Cross-talk between monocytes and NK-cells is important for viral clearance. Monocytes/macrophages respond to IFN- γ by activation. Following IFN- γ stimulation, resident macrophages present an increased phagocytic profile, necessary for successful viral elimination. As a result, reduced IFN- γ production by NK-cells prevents macrophage activation and promotes establishment of HCV infection.

T-cells consist of a heterologous population of CD4+ and CD8+ cells. CD4+ cells are further divided in T_{H1} and T_{H2} cells. These T-cell subsets have both morphological and functional differences. While T_{H1} cells produce IL-2 and IFN- γ , T_{H2} cells produce IL-4 and IL-5. Similarly, CD8+ cells are divided in cytotoxic type 1 (Tc1) and type 2 (Tc2) T-cell. Tc1 produce predominantly IFN- γ , whereas Tc2 produce principally IL-4, exerting an anti-inflammatory effect. There is also a subset of T-cells called Tregs that suppresses the activity of other subsets. Tregs produce and secrete IL-10, IL-4 and TGF- β (Dolganiuc & Szabo 2008). Evidently, T-cells are capable of modulating the activation and function of monocytes/macrophages through this battery of secreted cytokines. HCV infection favors the production of an altered T_{H1}/T_{H2} ratio of cytokines in addition to an increased number of Tregs (Bowen & Walker 2005, Taams *et al.* 2005, Tiemessen *et al.* 2007). This may lead to impaired activation and uninhibited phagocytic activity of macrophages, resulting in liver damage.

HCV persistent infection leads to chronic inflammation

Despite the combined efforts of innate and adaptive immune responses to eliminate HCV, in 80% of cases a persistent infection is established because the virus has come up with immune evasion mechanisms. Clearance of HCV by the immune system requires a strong and multi-specific T-cell response, by both CD4+ and CD8+, in combination with B-cell participation (Elliot *et al.* 2006, Koziel 2005). Additionally, a repertoire of innate immune cell subsets, like monocytes, macrophages, NK-cells and DCs is essential for a successful immune response (Szabo *et al.* 2007). Failure to mount such a response, fatally leads to persistent infection.

HCV has evolved ways to escape innate immunity both at the cellular and molecular level (Heydtmann 2009). Briefly, at the molecular level, HCV proteins succeed in disrupting TLR signaling, which has already been discussed, as well as the retinoid acid inducible gene I (RIG-I) pathway. The RIG-I pathway plays a key role in HCV infection by medi-

ating increased antiviral IFN- β production, as well as the induction of hepatic apoptosis via TNF- α /TRAIL (tumor necrosis factor-related apoptosis inducing ligand). NS3 and NS4A have been proposed to cleave interferon-beta promoter stimulator 1 (IPS-1), a downstream molecule in the RIG-I pathway, disrupting IFN- β induction (Horner & Gale 2009, Keller *et al.* 2007, Liu & Gale 2010, Szabo *et al.* 2007). At the cellular level HCV affects almost all cell subsets involved in innate immunity, predominantly by interfering with their signaling molecules, cytokines and chemokines. Finally, HCV evasion of adaptive immune responses includes a variety of strategies, such as escape mutants, T-cell anergy and exhaustion, and Tregs prevalence (Bowen & Walker 2005); however these will not be discussed, as they are beyond the scope of this review.

The heightened immune response results in constant secretion of inflammatory cytokines and chemokines and chronic inflammation is established. The ensuing accumulation of phagocytic cells, like macrophages and cytotoxic cells in the liver leads to tissue damage, which is key event in HCV infection. The immune system not only fails to eliminate the virus, but it also attacks “indiscriminately” both the healthy and infected hepatocytes, destroying in this way the patient’s liver.

The clinical manifestations of the HCV-induced chronic inflammation include development of fibrosis which may progress to steatosis, cirrhosis and/or malignant transformation. The development of liver fibrosis is the net effect of repeated cycles of tissue damage and repair, caused by HCV-induced chronic inflammation (Figure 3). HCV-infected hepatocytes release ROS and fibrogenic cytokines, like TGF- β and IL-8. This leads to activation of inflammatory cells and to further recruitment of immune cells. The activated immune cells trigger hepatic stellate cells to secrete collagen and in turn, they release cytokines and chemokines, which leads to additional recruitment of immune cells (Neuman *et al.* 2008). This vice circle results to an unrepaired hepatic injury and replacement of hepatocytes with extracellular matrix (collagen I, III, and IV, fibronectin). It has been reported that in advanced fibrosis stages, there is up to six times more extracellular matrix than in a healthy liver (Bataller & Brenner 2005).

Chronic inflammation in HCV persistent infection leads to cancer

HCC is the fifth most common cancer and the third leading cause of cancer death worldwide, mainly because of its poor diagnosis and high rate of recurrence.

Hepatocarcinogenesis is a multistep process that involves a number of genetic and epigenetic alterations, the activation of cellular oncogenes and/or the inactivation of tumor suppressor genes, and the deregulation of multiple signal transduction pathways. Furthermore, the role of hepatic immune-mediated chronic inflammation and its associated oxidative stress and accompanying potential for cellular DNA damage are unquestionably important contributing causes to carcinogenesis (Mazzanti *et al.* 2008). ROS can provoke DNA damage, leading to mutations. HCV has been proved to increase ROS production, increasing the possibilities for cancer cell generation (Aggarwal *et al.* 2006). In parallel, it has been suggested that HCV core and NS3 proteins impair the function of DNA repairing enzymes by binding to Nijmegen breakage syndrome 1 (NBS1) protein, which is important for the repair of DNA double strand breaks, in both hepatocytes and mononuclear cells (Machida *et al.* 2010). This finding provides a new insight for HCV ability to cause lymphoproliferative disorders, in addition to the hepatocellular carcinoma. Notably, in contrast to Hepatitis B virus (HBV), HCV does not integrate its genetic material to cell chromosomes, but it exerts its tumorigenic actions through viral-host protein interactions (Levrero 2006).

To date, there is a strong correlation between HCV-induced chronic inflammation and progression to cancer. Several cytokines and chemokines that take part in the inflammatory response against HCV are implicated in tumorigenesis. Pro-inflammatory cytokines have been linked to almost all steps of tumor progression such as cellular transformation, survival, proliferation, angiogenesis and metastasis. IL-6, IL-1 but above all TNF- α , have been found to possess tu-

morigenic properties. Particularly TNF- α was found to be a thousand times more efficient carcinogen than chemical carcinogen compounds. Furthermore, chemokines and chemokine receptors, like IL-8, MIP-1 β , RANTES, CCR7 and CXCR4, have been found to promote metastasis of cancer cells, due to their chemotactic properties (Aggarwal *et al.* 2006).

Activation of the inducible transcription factor nuclear factor- κ B (NF- κ B), which is constitutively expressed in cancer cells, is important for tumor progression (Ben-Neriah & Karin 2011). NF- κ B mainly mediates TNF- α and other pro-inflammatory cytokine action and production, since NF- κ B binding sites are present in the promoters of almost all cytokines and chemokines. Core has been suggested to be a potent regulator of NF- κ B activation (Mann *et al.* 2006, Ray *et al.* 2002). Additionally, NF- κ B controls the transcription of other genes important in tumorigenesis like cyclooxygenase-2 (COX-2), an enzyme involved in tumor progression, matrix metalloproteinase-9 (MMP-9), an extracellular matrix protein important for cellular migration, and activation-induced cytidine deaminase (AID), an enzyme responsible for chromosomal mutations (Aggarwal *et al.* 2006). HCV NS3 has been found to enhance COX-2 promoter activity in HepG2 cells (Lu *et al.* 2008). Additionally, AID has been found up-regulated in liver biopsies of HCV patients (Kou *et al.* 2007) and it has been suggested that HCV core is responsible for transcriptional activation of its promoter (Chiba & Marusawa 2009). Finally, HCV up-regulates MMP production in the liver, creating a favorable substrate for metastasis (Coenen *et al.* 2011, Okamoto *et al.* 2010).

Finally, HCV contributes to carcinogenesis through interaction of its proteins (core, NS3, NS5A)

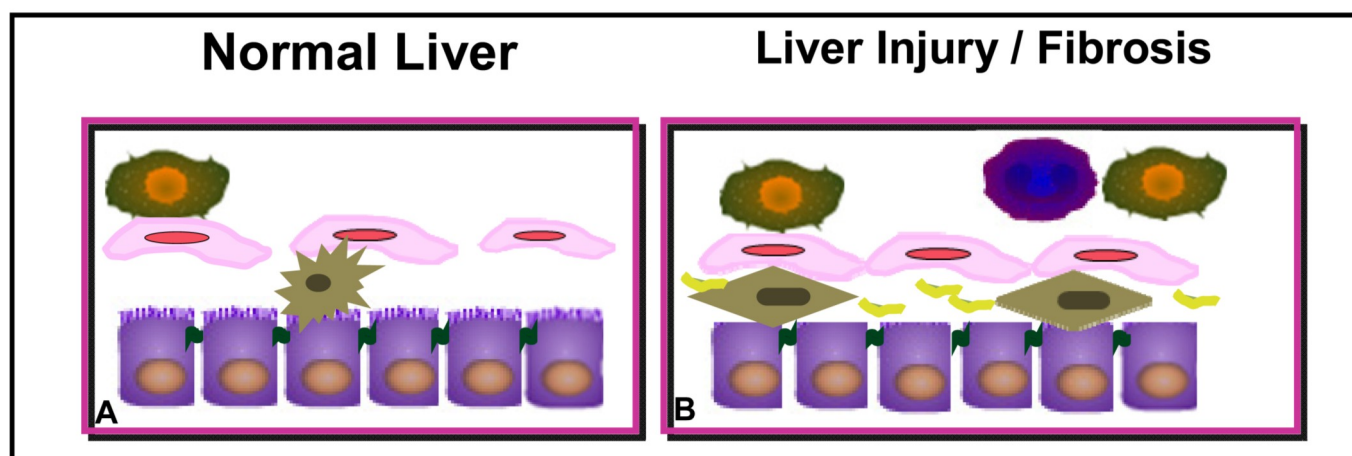


Figure 3. Alterations during hepatic fibrosis. Panel A represents a normal hepatic sinusoid. In Panel B loss of hepatocyte microvilli, comes as a result of stellate cells' activation and accumulation of extracellular matrix. Hepatocytic function is deteriorating. Kupffer cells are activated and monocytes migrate to the site of liver injury, thus contributing to the manifestations of inflammatory response. [Key: Hepatocyte (purple), stellate cell (brown), endothelial cell (pink), Kupffer cell (green), monocyte (round purple), extracellular matrix (yellow)]

with the master tumor suppressor TP53. In addition, core protein has been shown to interact with p73, another tumor suppressor. Moreover, HCV NS4A and NS4B have been found to interact with p21, a regulatory protein of cell cycle, in a post-transcriptional level. These interactions impart a direct tumorigenic capacity on HCV (Anzola & Burgos 2003).

Conclusions

Overall, in the present review, the ability of HCV to interact with cells of the immune system, mainly macrophages and monocytes, and the subsequent modulation of the expression profile of specific key cytokines/chemokines are discussed. The altered cytokine/chemokine expression profile leads to impaired innate immune responses and priming of adaptive immune responses. This represents a significant part of the HCV strategy to evade the host immune system and establish persistent infection that leads to chronic inflammation. HCV-induced aberrant inflammation is responsible for development of liver fibrosis and hepatocellular carcinoma.

Acknowledgements

We thank Dr U. Georgopoulou for the critical reading of the manuscript.

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