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Immune modulation by the Hepatitis C virus core protein

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HCV-core mediated Immune modulation.

***Title*:**

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Abbreviations:

APC: Antigen-Presenting Cell. **DC**: Dendritic Cells. **ERK1/2**: Extracellular Signal-Regulated Kinase**.** **HCV**: Hepatitis C Virus. **HCVne**: HCV Non-enveloped particles. **IL**: Interleukin. **MAPK** : Mitogen-Activated Protein Kinase. **mDC**: Mieloid Dendritic Cells . **NFAT**: nuclear factor of activated T cells. **NLS**: nuclear localization signals. **PBMC**: Peripheral Blood Mononuclear Cells. **pDCs**: Plasmacytoid Dendritic Cells. **PPR**: Pattern Recognition Receptors. **SOCS**: suppressor of cytokine signaling. **STAT**: Signal Transducer and Activator of Transcription. **TGF-β**: Transforming Growth Factor.

ABSTRACT

Hepatitis C virus (HCV) infection is currently the most important cause of chronic viral hepatitis in the world and one of the most frequent indications for liver transplantation. HCV uses different strategies to evade the innate and adaptive immune response, and this evasion plays a key role in determining viral persistence. Several HCV viral proteins have been described as immune modulators. In this review, we will focus on the effect of HCV nucleocapsid core protein in the function of immune cells and its correlation with the findings observed in HCV chronically infected patients. Effects on immune cell function related to both extracellular and intracellular HCV Core localization will be considered. This review provides an updated perspective on the mechanisms involved in HCV evasion related with one single HCV protein, which could become a key tool in the development of new antiviral strategies able to control and/or eradicate HCV infection.

KEYWORDS

HCV, Immune Evasion, regulatory T cells, Immune Tolerance, exhausted phenotype

INTRODUCTION

The Hepatitis C virus (HCV) is currently the most important cause of chronic viral hepatitis in the world and one of the most frequent indications for liver transplantation (1, 2). Globally, HCV is estimated to infect 80 million people (3).

The immune response against HCV infection does not show a fixed pattern. During the acute infection, the virus triggers an immunological response mediated by $CD4^+$ and $CD8^+$ T cells. In most patients who recover, $CD4^+$ T-cell responses to HCV are polyclonal, vigorous, easy to detect and maintained indefinitely after recovery; while in chronically infected patients, the response is narrowly focused, weak and usually undetectable (4). Interestingly, studies about the effect of several viral proteins in immune cells have shed light on the variability of the immune response against HCV.

In this review, we will focus on the effect of HCV core protein on immune cell function. As infection of immune cells by HCV has been widely demonstrated by several authors (5-13), effects on immune cell function related to both extracellular and intracellular HCV Core localization will be considered.

HCV Core protein: Structure and pleiotropic functions

HCV core protein is a highly basic RNA-binding protein, which forms the viral capsid. The core protein is released as a 191 aa precursor of 23-kDa (P23); however, proteins of various sizes (17 to 23 kDa) have been also detected, among which the 21-kDa core protein (P21) is the predominant form (14). Three distinct domains can be distinguished by amino acid content and hydropathicity patterns (15). Domain 1 (D1) spanning the first 122 N-terminal residues constitutes a mainly hydrophilic and highly basic domain that contains numerous positive charges. It is mostly involved in RNA binding and nuclear localization, as suggested by the presence of three predicted nuclear localization signals (NLS) (16-18).

Domain 2 (D2) comprises the C-terminus of the mature core protein and contains a significantly lower proportion of basic residues. It is mainly hydrophobic and it is predicted to fold into α-helices (19). This region is responsible for core association to lipid droplets in mammalian cells and with ER membranes (19, 20). Biophysical characterization of core protein indicates that this domain is critical for the folding and oligomerization of the core protein.

Domain 3 (D3), which lies from residue ~175 to 191, is highly hydrophobic, is at the extreme C-terminus of the immature core protein and acts as the signal sequence for E1 cleavage (14, 15).

HCV Core protein assembly and RNA encapsidation

Core protein multimerizes to form the capsid shell, which protects HCV RNA genome while the virus passes from one cell to another, or from one person to another, being RNA encapsidation, the main role of HCV core protein. The basic residues in the N-terminus are critical for HCV core protein and RNA association (21, 22). Besides multimerization to form the capside for RNA binding and protection, HCV Core protein has potent nucleic acid chaperone properties and directs dimerization of HCV (+) RNA 3´ untranslated region *in vitro*. These findings suggest that RNA dimerization could promote RNA recombination, during RNA replication in the infected cells or in the HCV viral particle, and in this way, HCV Core protein would be implicated in the genetic variability of HCV and in its capability to evade the immune system or some antiviral therapies (22, 23). Indeed, Core has been shown to bind ribosomal RNA (21), tRNA (24) and HCV genomic RNA (23, 24). The only requirement being that the RNA contains a significant amount of secondary structure as well as the presence of G-rich moieties (25).

In mammalian cell lines, HCV Core protein, can be targeted away from the ER due to lack of specific cellular factors or the presence of inhibitory factors. Alternative localizations

of Core could be correlated with failure to assemble HCV capsids or virions in cultured Mammalian cells lines (22, 26-28). Related with these findings, naked viral core/nucleocapsids without a lipid envelope have been observed in the circulation of infected individuals (29).

Furthermore, by means of a baculovirus expression system, uptake of naked HCV core particles generated in the absence of other HCV proteins has been shown (30, 31). HCV nonenveloped particle internalization into hepatic cells via low pH-dependent clathrin-mediated endocytosis, and their localization to early endosomes and finally into lysosomes have been described. During this entry process, different mitogen-activated protein kinase (MAPK) cascades are activated. Specifically, activation of the extracellular signal-regulated kinase (ERK1/2) pathway has been described, an event that could be important in HCV life cycle, pathogenesis and immune activation or evasion of HCV infection (32). Several studies have demonstrated presence of unenveloped virus-like particles resembling nucleocapsid-like structures and HCV core protein in hepatocytes (30, 33). Interestingly, HCV core protein has also been detected in different nonparenchymal liver cells of HCV infected patients, such as lymphocytes as well as Küpffer, polymorphonuclear, pit, endothelial, stellate, and fibroblast-like cells. HCV Core protein was immunolabeled in the cytoplasm and the nucleus of these cells and it co-localized with large lipid droplets present in stellate cells and with collagen fibers in the extracellular matrix (34).

There is evidence indicating that HCV can replicate in $CD4^+$ T cells lines such as Jurkat and Molt-4 (5, 35), being able to infect peripheral blood mononuclear cells (PBMC) *in vitro* (36) and *in vivo* (12, 13, 37-39), as well as lymph nodes *in vivo* (40). We and others have detected HCV RNA in peripheral blood $CD4^+$ T cells from chronically infected patients (8, 13) and it has also been identified in B cells (41-43), dendritic cells (DC) (38), monocytes and macrophages (43-45). Interestingly, the presence of HCV-Core protein has been detected not only in the serum of infected patients (29), but also in nonparenchymal liver cells

including lymphocytes (34) and in peripheral blood $CD4^+$ T cells of HCV infected patients (46). These findings support the topic of this paper, which is focused on the effects of HCV core protein in immune cell dysfunction.

Modulation of immune cell function by HCV core protein.

Effects of HCV core protein on the Innate immune response.

NK cells

Several studies have revealed the importance of NK cells as key players in the course of HCV disease, and impaired activity of NK cells has been proposed as a mechanism contributing to viral persistence in HCV infection (47-49). In addition, long term exposure to the virus during HCV chronic infection, diminishes the number of NK cells (50), changes their phenotype and behaviour, showing a weakened cytotoxic activity (51, 52) decreased perforin expression (52), altered IFN-γ secretion, increased expression of the inhibitory receptors CD94/NKG2A and production of the inhibitory cytokines, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (50, 53-56).

HLA-E regulates natural cytotoxicity by NK cells by its interactions with inhibitory CD94/NKG2A receptors, Nattermann et al. have tested 30 HCV core-derived peptides for HLA-E binding and inhibition of natural cytotoxicity by NK cells. They have identified HCV core amino acids 33-45 as a peptide stabilizing HLA-E expression in K562 cells, thus inhibiting NK cell-mediated lysis. Blocking experiments confirmed that the inhibitory effect of peptide 35-44, on natural cytotoxicity, was mediated by CD94/NKG2A interactions and enhanced HLA-E expression. Furthermore, *in vitro* data were supported by the finding of enhanced intrahepatic HLA-E expression on antigen presenting cells in HCV infected patients (57).

Recently, Golden-Masson et al. have shown a correlation between the proportion of peripheral NK cells with a $CD56^{bright}$ immature/regulatory phenotype, and the quantities of

circulating HCV Core particles in acutely infected patients. *In vitro,* incubation with HCV Core protein, in the presence of IL-15, maintains a significant proportion of NKs in an immature/regulatory $CD56^{bright}$ phenotype. HCV Core protein exogenous effects in NK cells could be explained by the presence of gC1qR on NK cells and its capacity to bind HCV Core protein (56).

Another study has shown that endogenous expression of HCV Core protein in YT NK-like cell impaire their cytotoxic activity, associated with a decrease in perforin and granzyme B production. HCV core protein–transduced YT cells show reduced IFN-γ production as well as a decreased expression of the natural cytotoxicity receptor NKp46 (58).

These findings suggest that exogenous and endogenous HCV core protein may alter NK cell function inducing an anergic state (56, 58).

Indirectly, HCV Core protein causes a negative regulation on NK cells function by a p53-dependent MHC class I and TAP1 mediated increase in HepG2 cells. Thus, the elevated MHC class I levels induce negative signals that lead to inhibition of NK cytotoxicity. Interestingly, at least for the experimental situation of exogenous peptide loading, HCV core expression does not influence recognition by HCV-specific $CD8^+$ T cells (59).

Dendritic Cells

The question whether HCV alters DC function has been addressed by several groups. DCs constitute a bridge between innate and adaptive immunity, being the professional antigen-presenting cell (APC) that plays a dominant role in priming and maintaining strong T cell responses in HCV infection. During initial HCV infection, DC interaction with the complete virus and/or virus-derived proteins may contribute to the priming and maintenance of $CD4^+$ and $CD8^+$ T cell responses, which will later result in clearance or persistence of the infection. ,It has already been reported that DCs are infected with HCV *in vitro* and *in vivo*

(38, 60), thus the virus could impair DC ability to stimulate the proliferation of T cells. In addition, it has also been described that expression or uptake of HCV gene products such as Core, E1 and E2, do not affect maturation of monocyte-derived DC by lipopolysaccharide (61), but could hamper DC function.

Monocyte-derived DCs obtained from patients with chronic HCV infection, show a decreased expression of HLA-DR and CD86; reduced levels of IL-12p70 secretion and high levels of IL-10 production. Therefore, DCs display an impaired capacity to induce T cell proliferation and activation (62, 63).

Furthermore, it has been shown that DCs expressing HCV core or NS3 protein display an altered maturation and an impaired antigen-presenting function, rendering them unable to stimulate anti-HCV T-cell responses. HCV Core and NS3 proteins induce a DC phenotype characterized by increased IL-10 and decreased IL-12 production with not change on DC costimulatory molecule expression, suggesting that the mechanism underlying T cell inhibition could be related with a maturation defect and an abnormal cytokine production during alloantigen-induced T cell proliferation (64-66).

HCV core and NS3 proteins also increase IL-6 secretion by both immature and fully differentiated DCs, this may induce the differentiation or expansion of $CD4^+$ Th17 cells that could be implicated in liver injury related to the viral infection (67).

Several defects have been described in plasmacytoid DCs (pDCs) isolated from chronically HCV-infected patients. pDCs are less frequent in the circulation due to apoptosis and show a reduced capacity to produce IFN-α. The reduced frequency of circulating pDC have been shown to correlate with an increased plasma level of HCV core protein. It has been suggested that HCV core-induced IL-10 and TNF-α in monocytes mediates these pDCs defects (68).

In addition, HCV Core protein has been described as a pattern recognition receptors (PPR) signalling modulator in pDCs, and its capacity to suppress IFN induction has been

linked with decreased IRF-7 protein levels and increased non-phosphorylated signal transducer and activator of transcription-1 (STAT-1) protein (69).

Professional Antigen-presenting cells, are not the only cells modulating immune responses in HCV infection, as hepatocytes that interact with T cells participate in immunological mechanisms within the liver. HCV core protein, either added to cell cultures or intracellularly expressed by mouse APCs [including murine hepatoma cells, freshly isolated hepatocytes or Mieloid Dendritic Cells (mDC)], downregulates MHC-I surface expression on all cells investigated. Specifically on Mieloid DCs (mDCs), HCV core protein reduces the expression of MHC-II and costimulatory molecules, and the secretion of IL-6 and IL-12. $CD4^+$ and $CD8^+$ T cells primed by these APCs display defects in proliferation, reduced surface expression of activation markers, decreased pro-inflammatory cytokine production and, interestingly, show an increase in IL-10 production, resembling the phenotype of regulatory T cells (70).

Monocytes/Macrophages

Eisen-Vandervelde et al. demonstrated that interaction of extracellular HCV core and gC1q complement receptor on the surface of human monocytes/macrophages suppresses IL-12 production (71). IL-12, which is known to regulate pro-inflammatory Th1 responses by promoting IFN-γ production, could be considered as an essential link between innate and adaptive immunity (72). These results are in agreement with clinical observations in HCV chronically infected patients, who show a diminished capacity to upregulate IL-12 and IFN-γ secretion and to generate Th1-type immunity (73). Supporting these findings, several studies have suggested that monocytes can be infected by HCV (43-45), and intracellular expression of a vector containing HCV core protein in activated murine macrophages severely diminished IL-12 and nitric oxide production (74).

Another work describes that HCV core and host derived factors, such as IFN-γ and endotoxin, favour the loss of TLR tolerance and contribute to an excessive TNF-α production in monocytes, inducing and maintaining monocyte/macrophage activation, thus favouring persistent inflammation in patients with chronic HCV infection (75).

To characterize the molecular basis for the regulation of APC function by HCV core, researchers examined the ability of extracellularly added HCV core to activate STAT family members (STAT1, -2, -3, -5, and -6). The study reported that HCV core-induced STAT3 activation is dependent on the PI3K/Akt pathway and requires production of IL-6. These results suggest that HCV core-induced STAT3 activation plays an important role in the alteration of inflammatory responses by APCs, leading to impaired anti-viral T cell responses during HCV infection (76).

HCV core protein effect on the adaptive immune response.

T Cells

HCV-core exerts inhibitory functions in T cells, leading to altered T cell function and/or proliferation. T cell function in the presence of core resembles the actual unresponsiveness of T cells in HCV chronically infected patients.

$CD8^+$ T cells

$CD8^+$ T cell response is defective in HCV chronically infected patients. Some reports have described the inhibitory effect of extracellular HCV core protein interaction with the complement receptor gC1qR, expressed on the surface of naïve T cells, showing that this binding results in suppression of $CD8^+$ T cell activation and proliferation (77). Extracellularly added core also led to a decrease in IFN-γ production in these cells (78).

Lukens et al. generated a transgenic mouse where the expression of HCV Core was directed to the liver. The effector function of $CD8^+$ T cells isolated from livers of HCVcore

transgenic mice, was impaired, with suppression of IFN-γ, TNF-α and granzyme B production. Interestingly, an increase in PD-1 expression by specific $CD8^+$ T cells and its ligand B7-H1 on liver DCs following infection with a recombinant adenovirus to deliver antigen, was described. Blockade of PD-1 pathway enhanced $CD8^+$ T cell effector functions. These results suggest that regulation of PD-1 pathway is essential for HCV core-mediated impairment of T cells responses and viral persistence in the liver (79). These findings are in agreement with data obtained from chronic HCV infected patients, where HCV specific $CD8^+$ T cells display an exhausted phenotype with high levels of PD-1 expression (80) and decreased CD127 expression (81). In addition, PD-1 expression is upregulated on T cells from healthy donors when they were incubated with extracellularly added HCV core. This upregulation is mediated through interaction with gC1qR, and led to an altered T cell activation, proliferation and apoptosis (82).

$CD4^+$ T cells

$CD4^+$ T cells have been described to be affected by both, the presence of intracellular HCV core as well as by extracellular addition of the protein. Direct binding of HCV core to gC1qR on $CD4^+$ T cells has been shown to inhibit T lymphocyte proliferation (83).

A highly differentiated population of $CD4^+CD25^+$ regulatory T cells have been described in chronic hepatitis C patients (84, 85). $CD25^{high}$ and $CD25^{int}CD4^+$ T cells isolated from chronically infected HCV patients, express Foxp3, CTLA-4, and can inhibit $CD25^-CD4^+$ T cells after stimulation with mitogen and interestingly after specific activation with HCV core. Furthermore, it has been shown that *in vitro* stimulation with HCV core protein increases $Foxp3^+CD25^{high}CD4^+$ and $Foxp3^+CD25^{int}CD4^+$ Treg cell subsets isolated from patients with chronic Hepatitis C. Treg cell clones from chronic hepatitis C and self-limited HCV infection show differences. Treg cell clones express lower CD25 and CTLA-4, are not active in autologous suppression assays and do not respond to HCV core in self-

limited HCV infection, probably because HCV core-specific Treg cells have disappeared after antigen elimination. In contrast, Treg-cell clones from chronic hepatitis C patients inhibit proliferation of autologous Th1 and Th2 clones in a dose-dependent manner upon mitogenic stimulation and are specifically activated by recombinant HCV core in an HLA-DR restricted fashion (86).

Inhibitory effects have been described with intracellular core expression as well. Bergqvist et al. showed that HCV core expression in $CD4^+$ T cell lines led to an increase in intracellular calcium levels (87) and nuclear factor of activated T cells (NFAT) activation after stimulation with PMA (88).

In accordance with these data, we and others have shown that HCV Core protein expression is sufficient to activate NFAT causing its translocation to the nucleus, as well as to differentiate Jurkat cells into an 'anergic-like' phenotype (8, 89). Intracellular HCV core expression inhibits $CD4^+$ T cell proliferation confirming the induction of clonal anergy by the intracellular expression of core in $CD4^+$ T cells (8, 89).

The expression of core protein in stably transfected T cells induces a decrease in IL-2 promoter activity and IL-2 production in response to T cell receptor triggering, while increasing secretion of other cytokines such as IL-4, IL-10, INF-γ and TNF-α (89). We showed that HCV core protein is able to induce *de novo* generation of $Foxp3^+$ T cells, inducing regulatory function. Using a third-generation lentiviral system to express HCV core protein in $CD4^+$ Jurkat T cells, we described that HCV-core-expressing Jurkat cells show an up-regulation of Foxp3 and CTLA-4 (90). The implication of NFAT in CTLA-4 expression is consistent with previous results, showing an activation of NFAT by HCV core and an implication of NFAT in anergy induction (8). In addition, we found that HCV-Core transduced Jurkat cells are able to suppress $CD4^+$ and $CD8^+$ T-cell responses to anti-CD3 plus anti-CD28 stimulation, suggesting that the high levels of Treg-cells described in peripheral blood from chronically infected patients may be developing at least partially in the periphery

upon internalization of viral particles or proteins (90).

Another researcher have described that HCV Non-enveloped particles (HCVne) can efficiently bind, being internalized in T cell lines and changing peripheral $CD4^+$ and $CD8^+$ T cell phenotype and function. In this study, peripheral T cells in the presence of HCVne, showed an elevated expression of CD127 and CTLA-4, compared to the respective control group. $CD4^+CD25^{+hi}CD127^-$ regulatory T cells and $Foxp3^+CD4^+$ T cells differentiation were also described. A significant transcriptional activation and increased secretion of TGF-β and IL-10, as well as, an IFN-γ transcriptional activation delayed with respect to that of IL-2 is compatible with these cells being partially exhausted (91).

We have shown that $CD4^+$ primary T cells lentivirally transduced with HCV core protein not only acquire an anergic phenotype characterized by an increased expression of CD25, CTLA-4, Foxp3 and a decreased expression of CD127, but also inhibit IL-2 production and proliferation of bystander $CD4^+$ or $CD8^+$ T cells in response to anti-CD3 plus anti-CD28 stimulation. We reported that HCV core is able to induce in $CD4^+$ primary T cells an increased basal secretion of the regulatory cytokine IL-10 and a decrease of IFN-γ production upon stimulation. HCV core induction of $CD4^+CD25^+CD127^{low}PD1^{high}TIM3^{high}$ regulatory T cells with an exhausted phenotype and decreased CCR7 expression was also described. The CCR7 diminished expression levels explain the HCV core induced-Treg cells sequestration in inflamed tissues, such as the infected liver (46).

Otherwise, some reports in the literature attribute an apoptotic role to HCV core in $CD4^+$ T cells. In a transgenic mouse model expressing core protein, it is observed that *in vivo* antigenic stimulation triggers a marked influx of HCV Core- expressing, antigen-specific, transgenic $CD4^+$ T cells into the liver. These cells overexpress FasL and induce liver inflammation (92).

Thus, the expression of core in T cell lines or in transgenic mice led to an increase in apoptosis, an altered T cell response and into the liver, could be related with liver damage

development (92, 93).

B cells

It has been demonstrated that HCV core protein up-regulates the expression of CD69 activation marker, promotes B cell proliferation and increases immunoglobulin M and immunoglobulin G production as well as cell surface expression of costimulatory and chemokine receptors, including CD86 (B7-2), CD154 (CD40L) and CD195 (CCR5), in $CD20^{+}$ B cells, while T cells exhibit a suppressive pattern, with decreased CD69 expression and diminished IFN-γ production. Interestingly, B cell activation by HCV core was associated with down-regulation of suppressor of cytokine signalling-1 (SOCS-1) expression and up-regulation of STAT-1 phosphorylation, while T cells suppression by HCV core exhibited the opposite pattern. In this way, chronic antigen exposure to circulating HCV core, while dysregulates T-cell responses and promotes an anergic state via up-regulation of SOCS proteins, also leads to down-regulated SOCS-1 and/or SOCS-3 expression in B cells. This might drive B-cell clonal expansion that is currently thought to cause development of mixed cryoglobulinemia and non-Hodgkin lymphoma in HCV cronically infected patients (78).

Otherwise, results obtained by microarray analysis of serial RNA samples from B cells that were infected with adenovirus harbouring full length HCV core protein, found that HCV core protein inhibits apoptosis, downregulates MHC class II expression but it does not alter expression of immunoglobulin genes in B lymphocytes. In this way, HCV core protein hampers the antigen-presenting function of B cells, but not the Immunoglobulins production (94).

Concluding remarks

HCV core protein plays a key role in immune system regulation during HCV chronic infection. In this review, we have summarized the current knowledge about the effect of

HCV nucleocapsid core protein in the function of immune cells, and its correlation with the findings observed in HCV chronically infected patients. The mechanisms involved are currently under investigation and they could become one of the key tools in the development of new antiviral strategies able to control and/or eradicate HCV infection.

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