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Hepatitis C Virus' initial encounters: mechanisms of innate immunity

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TABLE OF CONTENTS

1. ABSTRACT

HCV is a single-stranded RNA virus that affects approximately 210 million people worldwide causing chronic disease in 80% of those infected. With the development of new models of study, and a better understanding of the innate immune response, the way this virus induces and evades an immune response at the beginning of the infection has started to be recognized as a critical stage for the development of the chronic state. Still, even with so much information, the question remains as to how the virus establishes itself successfully in the host; a critical question that can lead to life-saving answers. In this review we aim to understand the initial interaction of the virus with its host based on the current literature that links innate immunity and HCV's effects over it.

2. INTRODUCTION

In 1989 a virus was discovered to be the cause of the blood-borne Non-A Non-B hepatitis infection that can lead to liver disease (1, 2). This member of the family *Flaviviridae*, later termed Hepatitis C virus (HCV), infects approximately 210 million people worldwide out of whom 80% will develop persistent infection (3, 4). This chronicity appears to be, at least partially, controlled by the host's immune response to the virus. It is the reason why therapies aimed at it, including the current one (pegylated IFN/Ribavirin), often target the prevention of replication while jump-starting the immune response to induce clearance. However such therapies have proven ineffective in 50% of patients, which highlights the need to understand the precise molecular mechanism behind these therapies

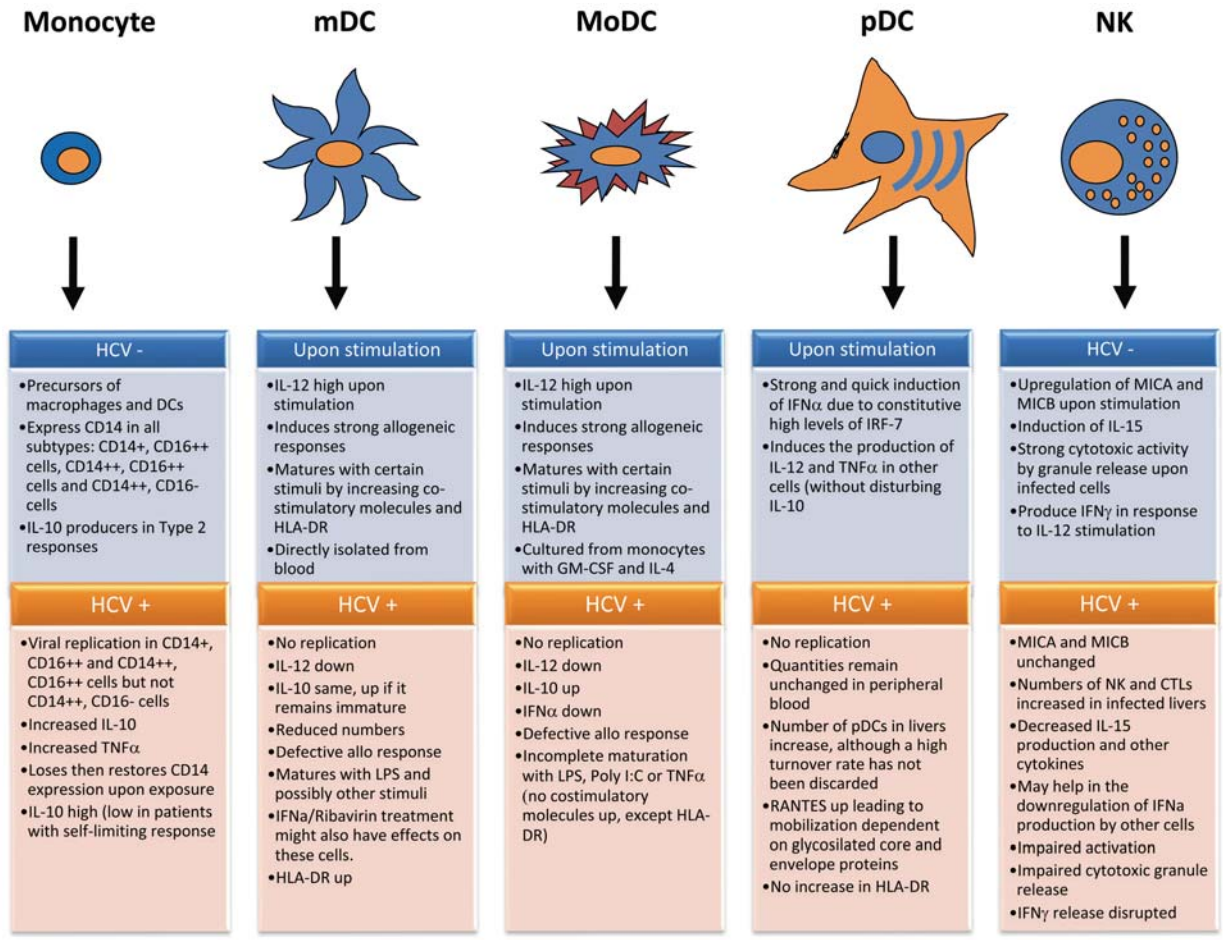

Figure 1. Comparison of the effects of HCV on DCs and related innate immune cells. The table represents a compendium of observations made either on clinical samples or cells from *in vitro* experiments. Each column is represented by a representative cartoon of each cell type.

and the reasons for non-response (5-7).This review aims to understand the virus' initial interaction with its host both at the cellular and receptor levels and the direct consequences of the initial host-pathogen interaction.

3. CELLULAR INNATE IMMUNITY IN HCV

Several lines of evidence point at the importance the type of immune response plays in preventing or maintaining the chronic state of HCV. For instance, a bias towards type 2 responses has been shown to favor a chronic state in many diseases, including HCV (8-11). *In vitro* studies have further confirmed this for HCV by showing that PBMC stimulated with viral proteins are induced to secrete IL-10 and inhibit IL-12 (and hence IFN gamma as well) (10, 12). Furthermore, one of the mechanisms of action of the IFN/Ribavirin therapy is the suppression of IL-10 without affecting type 1 cytokines (13). It is also becoming increasingly clear that the initial interaction of the virus with both immune and non-immune cells holds the key to understanding the reason for such adaptive immune shifts and viral escape. In PBMC, the likely culprits for the shift in the T_H1/T_H2 balance are dendritic cells (DCs) as demonstrated by several observations: 1) DC have been found critical in directing the type of the antiviral response in chronic infections; 2) HCV patients with specific MHC II haplotypes are more likely to clear the infection; 3) A defective IL-12 production from patient's DCs was responsible for a defective antigen presenting response and IFN gamma production; 4) Reduced DC percentages in HCV patients (although this can also be attributed to liver disease since there is an inverse correlation with serum ALT concentrations, Figure 1)(14-21).

3.1. Impact of HCV on DCs

Current research seems to drive the hypothesis that viral binding and/or entry (without the need for replication) can cause a myriad of "defects" in these cells (22, 23). These are thought to contribute to the chronic state since clearance of the virus, either spontaneously or by therapy, restore cells to their normal state. Those defects include: low *in vitro* stimulatory capacity of patient DCs, lower expression of co-stimulatory molecules, decreased IL-12 secretion (with no change in IL-10), decreased type I IFN and interferon stimulated genes (ISG) expression and,

as mentioned before, low absolute DC numbers in the periphery (14, 17, 20, 22). Apart from these characteristics, these cells show no apparent differences in phenotype and functionality as compared to healthy controls (24, 25). Furthermore, these characteristics are virus-specific since the cells retain their ability to respond to other antigens or to respond to the direct addition of activating cytokines, like IL-2 or IL-12 (26-29). Furthermore, pulsing cultured patient DCs with certain viral proteins correlates with the levels of IL-10 and TNF alpha as well as with the down-regulation of the expression of proteins in the NF-kappa B pathway (10, 12, 30). Therefore, the next question is to determine if this is a direct consequence of viral replication and at what stage it occurs.

Several experiments have suggested the possibility that HCV does not replicate in DCs and that they are used instead by the virus to gain passage to the liver since they have been shown to carry viral RNA that clears when they are placed in culture (24, 25, 31). Final confirmation came after the discovery that Claudin-1 was necessary for entry since DCs do not express it (32). It was then demonstrated that even pseudo-particles expressing E1 and E2 are unable to enter. Therefore, it is currently understood that HCV does not replicate in these cells, indicating that the effects caused by the virus are a consequence of binding to DCs. DCs do express some of the binding receptors for the virus and viral particles have been shown to be internalized, leading to viral protein presentation (33-35). Our own research actually demonstrates that viral envelope proteins could have a modulatory effect themselves by downregulating the expression of TLR3, such as has been demonstrated for TLR7 by Chang *et al.* (Eksioglu EA *et al.* submitted for publication and (36)). A likely scenario is that DCs are an intermediate that deliver the virus from the periphery to the liver since the HCV particles inside DCs are targeted to the non-lysosomal compartments where they are protected from degradation (31, 37). Conversely, these effects are not equal among DCs and illustrate the importance of understanding both DC subsets as well as other cells involved in viral innate recognition in more detail.

3.1.1. Myeloid and monocyte-derived DCs

Most of the research on DCs in the context of an HCV infection has been carried out on monocyte-derived DCs (MoDC) for their ease of use or from the isolation of total DC populations from peripheral blood. Most of these cells were derived directly from patient samples and it was not until recently that the discovery of the HCV strain JFH-1, provided the capability to study the direct interaction of the virus with these cells (38, 39). These observations on total DCs from chronic patients indicated that the phenotypes observed correlate strongly with those described earlier (15, 19, 25, 40-43). Interestingly, HCV-mDC and HCV-MoDC differ in their ability to react to maturation stimuli. LPS improves the allostimulatory capacity of mDCs, although not completely, which led many to the conclusion that there are no defects in these cells (23, 28, 29). MoDC from HCV patients do not behave this way and seem to remain partly immature after LPS maturation, likely due to an increase in IL-10 combined with a decrease in IFN alpha (25, 31, 44). Similar results are observed with other maturation stimuli like Poly (I:C) or TNF alpha (10). In general it seems that the immature cells are unable to up-regulate co-stimulatory molecules as compared to non-HCV DC and this may account for the lack of allo-stimulatory reactivity observed in these cells. More insightful information has been recently obtained *in vitro* with the aid of JFH-1 co-cultured with healthy DCs. The virus directly inhibited the up-regulation of co-stimulatory molecules without changing the expression of HLA-DR (31, 45-47). Interestingly, the sera of HCV patients were able to reduce the latter when added directly to cultured MoDCs (31, 47). In this setting pro-inflammatory cytokines were also absent while IL-10 increased, mimicking what was seen *in vivo*. JFH-1 by itself was not able to inhibit LPS-induced maturation or cytokine production, in contrast to what was observed in MoDC derived from chronic patients (23, 28, 29, 45). We ourselves have studied the interaction of MoDC with JFH-1 at different stages of differentiation (monocytes, immature, LPS-matured) (46). We observed that monocytes do not differentiate into DCs upon stimulation with HCV and immature DCs remained low in co-stimulatory molecules, similarly to what has been observed by others. Interestingly, the effect on mature DCs induces a shift towards a type 2 response with higher IL-10 after infection. These effects happened in the absence of viral replication. Most groups assumed that there were no effects on these cells due to the lack of phenotypical changes, but instead they may be part of the inducers of a suitable environment for the virus.

Transfection experiments have also hinted at the possibility that viral proteins themselves (core, NS3 and to a lesser extent the envelope proteins E1/E2) may be important in inducing the phenotype observed on mDCs, perhaps by recognition through TLR2 (30, 47-52). Some of this research had been carried out when trying to harness the immunogenicity of these proteins in order to use loaded DCs in therapies against hepatitis C. In this particular setting it was realized that while pro-inflammatory responses were induced, IFN alpha was severely impaired which had nothing to do with the maturation stage of these cells (10, 47, 49-54). The normal phenotype was recovered once cells were treated with IFN alpha this may shed light on the reason behind the disparity observed in clinical samples of mixed DCs (27, 47, 48, 55). It may be that these proteins stimulate directly through TLR2 inducing the secretion of IL-10 which may induce some of the phenotypes in mDCs and MoDCs (56). Furthermore expression of TLR2 as well as MyD88 is enhanced in mDCs from HCV patients while preventing the induction of cytokines from these receptors (40). This over expression might also be induced by endogenous IFN alpha expression in these cells. Other TLRs do not seem to be involved in this pathogenesis but are capable of inducing others. For instance, TLR7/8 agonists impair monocyte-derived DC differentiation and maturation and, furthermore, the phenotype of TLR7/8 ligand-treated DCs is similar to DC defects found in HCV-infected patients (30, 57). It seems clear that a better understanding of how innate receptors recognize and modulate the immune

response will be of the utmost importance in HCV and may carry over into other diseases as well.

3.1.2. Plasmacytoid DCs

The strength of this DC subset lies in its strong ability to produce vast amounts of type I IFN, especially IFN alpha, in a short period of time (58). These cells express constitutively express high levels of IRF-7 which is needed to produce the different types of IFN alpha (59). This cytokine alone can enhance the expression of HLA class I and class II molecules, co-stimulatory molecules on immature DCs (but not induce full maturation of mDCs which requires CD83) and induce IL-12 and TNF alpha without affecting IL-10 (13, 60). Interestingly, other reports have suggested that IFN alpha can have a negative effect on the induction of maturation of mDCs by some viruses; even though this has not been shown for HCV (42). This suggests that the IFN alpha/Ribavirin treatment might affect DCs and their function. Conversely, this same report also suggested that these observations might not be as relevant in this setting due to the fact that when combined with Ribavirin, they are known to enhance immune responses to HCV (61, 62). As a matter of fact responders in this treatment induce ISGs, like 2^05^0-oligonucleotide synthetase, MX1, IRF7, and TLR7 genes as compared to poor responders. Clinically, it appears that HCV does not affect the quantity of pDCs in peripheral blood other than affecting their total numbers (21, 42, 63). At the same time, an increase in the numbers of pDCs in HCV^+ livers is observed, most likely due to the upregulation of RANTES (18, 61, 64, 65). This mobilization can be induced by either E1/E2 or core protein interactions demonstrated by a need for glycosilated HCV proteins which are needed for IFN alpha induction in these cells (61). Conversely, IFN alpha production in these cells could be downregulated via the induction of IL-10 and TNF alpha by myeloid cells (monocytes and immature mDCs) either directly or by the induction of pDC apoptosis (64, 66). Furthermore, the increase on this cytokines correlated with their concentration in HCV patient sera (67, 68). Overall, it seems plausible that a subsequent downregulation of IFN alpha takes place, leading to many of the observations we described here. A lack of IFN alpha can induce DC survival as well as a maturation factor both of which are missing in HCV, including pDCs (63). Furthermore, although the overall levels do not change, it does not preclude a high turnover rate in these cells due to the decrease in IFN alpha and hence, the induction of apoptosis (64). Interestingly, contrary to mDCs and MoDCs, these cells did not increase HLA-DR after TLR-stimulation although co-stimulatory molecules did. Whether this occurs merely by a direct interaction of the virus with PRRs like TLR7 and 9 in pDC or by a bystander effect is not fully understood, but IFN alpha may be downregulated due to viral interaction with cell surface receptors. Interestingly, the data derived from patient cells did not correlate well with experiments carried out with JFH1 where the direct interaction of the virus with cultured pDC, and not IL-10 and TNF alpha produced by monocytes, was the culprit in the lack of IFN alpha response by these cells (45).

3.2. NK and cytotoxic cells

Recently a clear understanding of the mechanisms of DC have developed due to their their direct interaction with cells of cytotoxic potential such as $CD8^+CD28^-$ T cells, NK and NKT cells and their role in the pathogenesis of disease (45, 69). Their interaction with these cells leads to NK cell activation as evidenced by the upregulation of MICA/B in response to IFN alpha which is impaired in chronic HCV (70).The quantities of these cells in the liver also tend to increase while needed T_H cells as well as the ratio of CD4:CD8 T cells decreases (71). DCs can also be implicated in the downregulation of type I IFNs by HCV or be directly affected by diminished IFN production mediated by decreased IL-15 production (70, 72). In this case, evidence indicates that a direct interaction of HCV-E2 with CD81 in the NK cell can impair its activation, cytokine production, cytotoxic granule release and proliferation (73, 74). Furthermore, proliferation by these cells is impaired in HCV since NS5B modulates the cell-cycle progression in these cells leading to arrest (75). It is not clear whether a direct virus interaction or an interaction with affected cells (like DCs) diminish the activity of these cells but their cytotoxic potential makes them important targets for future studies, especially at the DC-NK interface and its role on HCV.

3.3. Hepatocytes as key inducers of innate immunity against HCV

Hepatocytes, the end host of HCV, are usually considered as a secondary in the immune fight against this virus as compared to immune residents of this tissue (76). A myriad of evidence is changing this image demonstrating that the initial innate response by liver cells can not only prevent viral infection, but also induce a state that would implement faster clearance in the event of infection, even if the majority of the peripheral IFN comes from immune cells (69, 76-78). Apart from their direct use against HCV, evidence for the importance of type I IFNs in the induction of an antiviral state in hepatocytes comes from *in vitro* as well as *in vivo* studies on chimpanzees (79). In both of these types of study, there seems to be a direct correlation between IFN and viral levels (79-81). Furthermore, the fact that both levels fluctuate in primates provides further evidence that the interaction of HCV with its host is more of an equilibrium that can change drastically under specific conditions. Both of these models lacked that critical evidence that would connect IFN with viral levels which illustrates the need to understand the underlying mechanisms of action in the body of the host.

Until recently, most of the data indicated that hepatocytes did not induce type I IFN in response to HCV. Further advances have demonstrated that this was probably due to the use of hepatoma cells as model systems since most of them have defects in the IFN pathway. For instance, Huh7 cells are poor producers of IFN due to the lack of TLR3, worsening with each passage, and Huh7.5 cells further lack RIG-I perhaps increasing viral efficiency (79, 80, 82-86). This view has come to change with the development of new liver cell lines (including one developed by our group named LH86 as well as immortalized non cancerous liver cells) highlighting the initial interaction of the virus and its probable role in the induction of IFN and its derived ISGs (75, 80, 87, 88). These new cells, combined with studies in primary

hepatocytes and animal models, will help illustrate the initial interaction of HCV with the liver and demonstrate the dynamic equilibrium that can lead to viral escape or clearance.

4. INTERFERON CASCADE IN HCV INFECTION: A KEY PLAYER

4.1. Early events in the type I IFN response

Most of the initial immune response is done through the recognition of the virus' genetic material by pathogen recognition receptors (PRRs), such as TLRs and cytosolic receptors like the DexH(D) RNA helicase, retinoic acid inducible gene-I (RIG-I), followed by the induction of the type I IFN responses (83, 89-93). This is an intrinsic system in all cells and constitutes the individual source of immunity against invading pathogens. The IFN cascade starts with binding to specific sites inside the receptor, such as the leucine-rich repeat motifs in the ectodomain of TLR3 or the helicase/ATPase domain of RIG-I (94-98). This interaction leads to the binding of adaptor molecules specific for each receptor initiating the cascade (TRIF and Cardif respectively) (91). These events converge at elements that are part of transcription factors in charge of genes related to the amplification of the IFN signal and initiate adaptive responses, known as IRF-3, NF-kappaB and ATF2/c-jun (94).

IRF-3 is constitutively expressed in cells were it awaits activation of the IFN cascade after pathogen recognition. It is activated by phosphorylation of its C-terminus which promotes its dimerization to other IRF-3 particles or to IRF-7 (99-102). In general, this phosphorylation is mediated by members of the non-canonical I kappa B kinases: IKK epsilon and TBK-1 (Tank binding kinase 1, also known as NAK for NF- kappa B activating kinase) (89, 99, 103). This event leads to nuclear translocation and association with CBP/p300 histone acetyl-tranferases binding on the DNA which transactivate the downstream genes of the early IFN response: IRF-7 (except on pDCs), IFN beta, IFN alpha 1 and RANTES (104). HCV's non-structural protein NS3/4A has been demonstrated to block this pathway (NS3 serine protease/helicase domain by itself is not enough) with subsequent redistribution from the cytoplasm to the nucleus (79, 80, 83, 91). These kinases do not seem to be affected by NS3/4A and furthermore, when overexpressed can affect viral replication (89). IKK epsilon's main role, on the other hand, may be in the induction of HCV-dependent apoptosis due to its recruitment to the mitochondria (89, 99). A second IKK complex is in charge of NF kappa B. This factor is normally repressed by I kappa B which gets removed by phosphorylation from the complex of IKK alpha, IKK beta and the regulatory subunit IKK gamma (NEMO) (105). This happens by an interaction with Cardif in the mitochondria (which does not happen to TBK1) or TRIF in the cytosol (91, 99, 106). The end goal is the nuclear translocation of NF- kappa B to initiate the transcription of pro-inflammatory genes. A third pathway is involved with the initiation of mitogen-activated protein kinases (MAPK) signaling cascades which leads to the activation of AP1 members. Some of these ISGs might also be induced by IRF-3 and NF- kappa B without the induction of the IFN pathway (89).

4.2. Late events in the type I IFN response

Recognition of the invading virus leads to a rapid cascade that ends with the induction of IFN beta and IFN alpha1. These cytokines then act in a paracrine fashion to continue on the enhancement of this innate response through the IFN receptor, formed from the dimerization of two components: IFNAR1 and IFNAR2. There is only one IFN beta but 11 subtypes of IFN alpha exist and are largely produced by immune cells whose particular purpose is not clearly understood (72). IFN beta itself can be subdivided into three pathways: 1) Insulin stimulation by IRS1 and 2; 2) MAP kinase and 3) ERK2 kinase. Later events in the response happen after IFN binds to the receptor just mentioned inducing a JAK/STAT signaling pathway which regulates the next step of the response by induction of ISGs including many pro-inflammatory cytokines (72, 80, 89, 99, 107, 108). HCV does not seem to directly interfere with this amplification part of the type I IFN pathway but disrupts the initial cascade involved in the production of IFN beta and the amplification loop (91). The end result is the reduced expression of IFNs and ISGs probably playing a role in the inefficient activation of the adaptive response to the virus.

4.3. Effects of the cascade and induction of adaptive immunity after HCV

One of the main consequences of the activation of innate immunity is the initiation of the adaptive response against the infection. In the case of HCV, a pro-inflammatory response is desired to induce viral clearance but it can also be the cause of pathology that leads to hepatitis. For instance, high levels of the pro-inflammatory cytokines IL-1 beta, IL-6 and TNF alpha are induced and strongly correlate with liver damage in chronic HCV (14, 81, 109, 110). These cytokines may not act directly on the virus but they increase the levels of certain PRRs, like TLR3, inducing high IFN which maybe the reason why there is an increase in this response which favors viral clearance (108). As a matter of fact, the only cytokine that seems to have a direct effect against viral replication *in vitro* is IFN beta (81, 88, 111). These cytokines are induced by TRAF6 activated transcription factor called IRF-5 which is also responsible for IL-12, IL-18 and cyclo-oxygenase 2 (102). Interestingly, IL-12 is not induced in HCV, but instead an increase in IL-10 is induced, which is better in terms of liver pathology, although at lower levels than IL-1 beta, IL-6 and TNF alpha (14, 81). Furthermore, the virus itself can mediate changes in their secretion to favor conditions for its propagation by inducing the secretion of IL-10 and TNF alpha by monocytes (Core and NS3)(14).

Other cytokines that are linked to liver injury are CXC and CC chemokines as they are also linked to the metastatic potential after transformation in chronic HCV (88). CCL3 is involved in hepatocyte inflammation (whose expression is increased in HCV infected patients and is linked with IFN non-responsiveness), CCL5 (also named regulated upon activation, normal T-cell expressed and

secreted RANTES), CXCL8 (IL-8) and CXCL10 (IFNg activated protein IP-10) (89, 112). Some of these chemokines are reduced by specific viral proteins because they are a direct consequence of the IFN pathway. For instance CCL5, CXCL10 and CXCL8 induced expression by Sendai virus-infection are reduced in response to full HCV genome or NS3/4A probably highlights the role of PRRs in the induction of these chemokines (76, 79, 88, 91, 104, 112). In contrast, some of the viral proteins like the structural proteins or NS5A, can actually increase the levels of some or all of these cytokines in a manner unrelated to the presence of viral RNA (104, 112).

4.4. Toll-like receptors in the recognition of HCV

To better understand the pathway choice and the role it plays in HCV, it is important to also understand the receptors themselves. Evidence suggests that the stage is set at the pathogen recognition level, more specifically with TLRs, which correlate with a decrease in the type I IFN response. For instance, while they do not completely abrogate the defects observed, different TLR ligands are capable or overcoming some of them, leading to DC maturation and activation of T_H cells in some cases (10, 63). The livers of chimpanzees experimentally infected with HCV have a high induction of type I IFNs and ISGs even at the incubation stage. The virus is able to evade, and in human's co-infection with GBV-C, seems to protect during co-infection due to the activation of the interferon system and the induction of maturation of DC (40, 113). Even more striking seems to be the fact that patients who have an increased expression of certain TLRs in their PBMC fared better in their response against the virus (114). Conversely, since most of these observations are clinical in nature they do not offer any insights to the initial interaction with the virus. It also does not take into consideration the distinct subpopulations of DCs which can respond to pathogens in varied ways, even inside the same host. The two subtypes are characterized by different functions and receptors: for instance, mDCs and MoDCs express TLR3 and home to lymphoid organs while pDCs express mainly TLR7 and TLR9 and are sometimes termed "professional interferon producers" due to their ability to produce close to 1000 times more type I interferon than any other immune cell (47, 58). In the liver TLR3 and TLR7 are expressed not only by resident DCs and other immune cells but by hepatocytes as well (36, 115-117).

TLR9 recognizes DNA material which is not part of the replicative cycle of HCV although it can be directly affected by HCV (45). TLR7 and 8 recognize guanosine or uridine-rich ssRNA but TLR8 does not induce IFN beta which is critical in inducing an antiviral state for this virus (92, 118, 119). They are also restricted in expression mostly to immune cells like pDCs. TLR3 recognizes double stranded RNA (dsRNA), including its synthetic analog polyinosinic–polycytidylic acid (polyI:C), by producing IFN beta and priming for an adaptive response (105, 120). This last part can be achieved through cross-priming by conventional DCs which lead to the activation of cytotoxic lymphocytes (CTLs) to clear virally-infected cells (121). These two receptors seem to be focused on different subsets of cells for the induction of type I IFN since TLR3 may be present in the surface of endosomes of many cells including hepatocytes (requiring acidification for activation in DCs), while TLR7 seems restricted to the endosomal compartments of pDCs (83, 108, 122). It seems that TLR3 is ubiquitously expressed in many non-immune tissues, which highlights its importance as a first line of defense against pathogens (120). Furthermore, both TLRs have been shown to induce a robust antiviral activity against HCV perhaps through structured dsRNA regions like 5' and 3' NTR (76, 81, 89). TLR3 also can recognize HCV-infected apoptotic bodies it ingests by fusion with TLR-3 containing endosomes inducing its maturation (69, 123). This function is particularly important for the recruitment of NK cells and CTLs. Therefore, most of the focus of TLRs in HCV has been on TLR3 and TLR7, although mostly in immune cells.

TLR3 and TLR7's role in liver cells or cell lines has not been deeply studied because of the use of hepatoma cells with defective IFN responses, as described earlier. Out of the functional model systems HepG2, HepaRG and LH86 cells have been shown to have active TLR3 and/or TLR7 pathways, not uncommon in tumor cells since these receptors may also be involved in tumor progression or apoptosis (87, 88, 110, 124). Immortalized human hepatocytes, like PH5CH8, also express TLR3 and further up-regulate it after poly (I:C) stimulation, just as cell lines with TLR expression do (75, 80, 108). Interestingly, in the case of chronically infected hepatocytes, the expression of these receptors has been shown to be down-regulated and to correlate with the dwindling levels of IFN□ or with poor responses to IFN treatment in these patients (14, 110, 116). In this instance, it is likely the action of NS3/4A itself (which we will describe in particular later) interferes with the IFN beta promoter downregulating the expression of several key components of the cascade (79). During HCV infection complications (such as glomerulonephritis, primary biliary cirrhosis or any type of liver inflammation) the virus seems to instead increase the expression of these receptors (108, 117). This disparity, and the actual role of the virus in it, requires further investigation. The *in vitro* correlation between IFNs, virus and PAMP expression could lead the way into a better understanding of what is happening in the chronically infected liver and the reasons why these pathways do not clear the virus *in vivo*.

4.4.1. HCV directly interferes with the TLR3 pathway

TLR3 uses TIR-domain containing adaptor protein-inducing IFN beta (TRIF, also called TIR-domain-containing molecule 1 or TICAM1) while MyD88 is the adaptor for most other TLRs (91, 94, 102, 125). Signaling leads to the eventual activation of IRF-3 and NF- kappa B (Figure 2). NS3/4A has been shown to directly cleave different adaptors for innate immune pathways with TRIF being one of them (between Cys-372 and Ser-373 which shares similarities with the NS4B/5A site on the HCV polyprotein) (79, 83, 91, 106). It was also demonstrated that the cleavage of TRIF impedes signaling by downregulating the levels of this adaptor and not due to any dominant negative activity by either section of the product (79). Interestingly, this was not observed in non-neoplastic PH5CH8 cells although it is not discounted that a balance

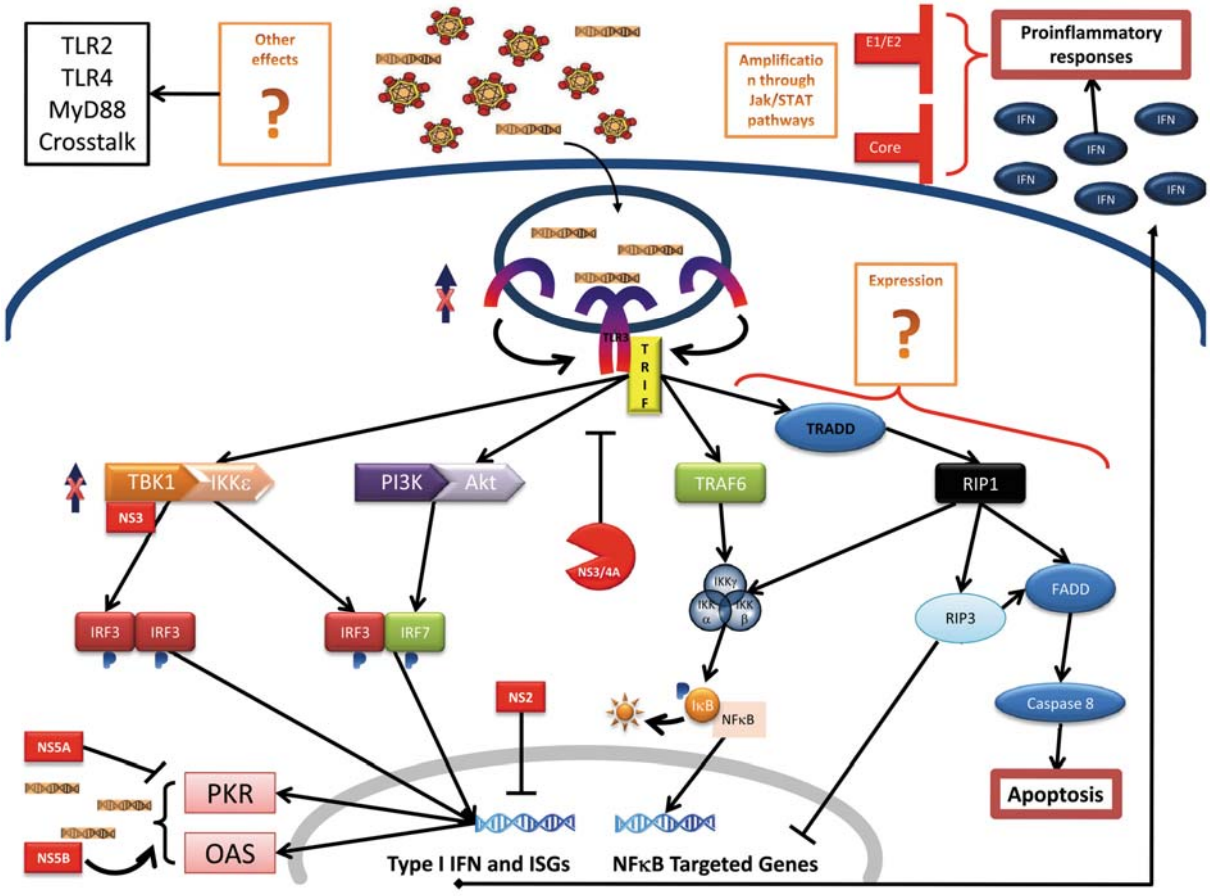

Figure 2. Schematic representation of HCV recognition and evasion by the TLR3 pathway. Recognition of extracellular HCV RNA by TLR3 occurs after ingestion of genetic material released by neighboring necrotic cells or by apoptotic bodies from infected cells. Once in the intracellular compartment binding of RNA to TLR3s ectodomain stimulates its dimmerization and recruitment of the adaptor TRIF. This is the main place of HCV interference with NS3/4A cleavage of TRIF which disrupts all the signaling from this point. Here the signal can be subdivided into four main paths: TBK1/IKK epsilon, PI3K, TRAF6, and TRADD/RIP1. The first two lead to the production of type I IFNs and ISGs by phosphorilating IRF3 and IRF7 molecules. HCV can downregulate the expression of TBK1 by an unknown mechanism while NS3 has also been shown to directly bind to it. There are no known interactions of HCV with PI3K. Downstream HCV can have effects on two ISGs: PKR and OAS which can also recognize viral RNA. NS5A downregulates PKR whiel NS5B (the replicase) can induce the production of IFN through either one. The other two lead to the phosphorilation of the IKKs which remove I kappa B from NF- kappa B and allow its translocation to the nucleus to induce pro-inflammatory genes. RIP1 has also recently been demonstrated to lead to the induction of apoptosis. The induction of IFNb can also lead to a feedback loop through the Jak/STAT pathway, that increments the IFN and proinflammatory responses. Structural proteins may have some modulatory properties, especially at the adaptive part of the response. The expression of some of these proteins is thought to be downregulated although further exploration in this area is required. On that subject TLR3 is also downregulated in the liver biopsies of HCV patients but it has not been demonstrated *in vitro*. There may also be effects that come from other PRRs or perhaps a crosstalk that leads to immunomodulation of these pathways.

in the levels of TRIF and the abundance of NS3/4A might play a role . In that light it is interesting to note that several HCV proteins (such as envelope, core and NS5B) tend to induce IFN beta which may or may not get downregulated by overexpression of NS3/4A (75, 91).

After TRIF, the signal can be subdivided into four different pathways with different outcomes. For the production of type I IFN the signal goes to either TBK1/IKK epsilon, or to PI3K which leads also to phosphorilation IRF-3 and IRF-7 making a heterodimer that induces IFN alpha (91, 100, 126-134). PI3K-Akt pathway expression (below TLR3-TRIF) is not able to specifically induce IFN beta promoter activity after dsRNA stimulation (91). For the induction of NF kappa B, the cascade can go to either TNFR-associated factor-6 (TRAF6) or to Receptor Interacting Protein-1 (RIP-1)(125). In chronic HCV patients the levels of IKKs expression are downregulated (89). Interestingly, over-expression of any of the IKK molecules before *in vitro* HCV infection seems

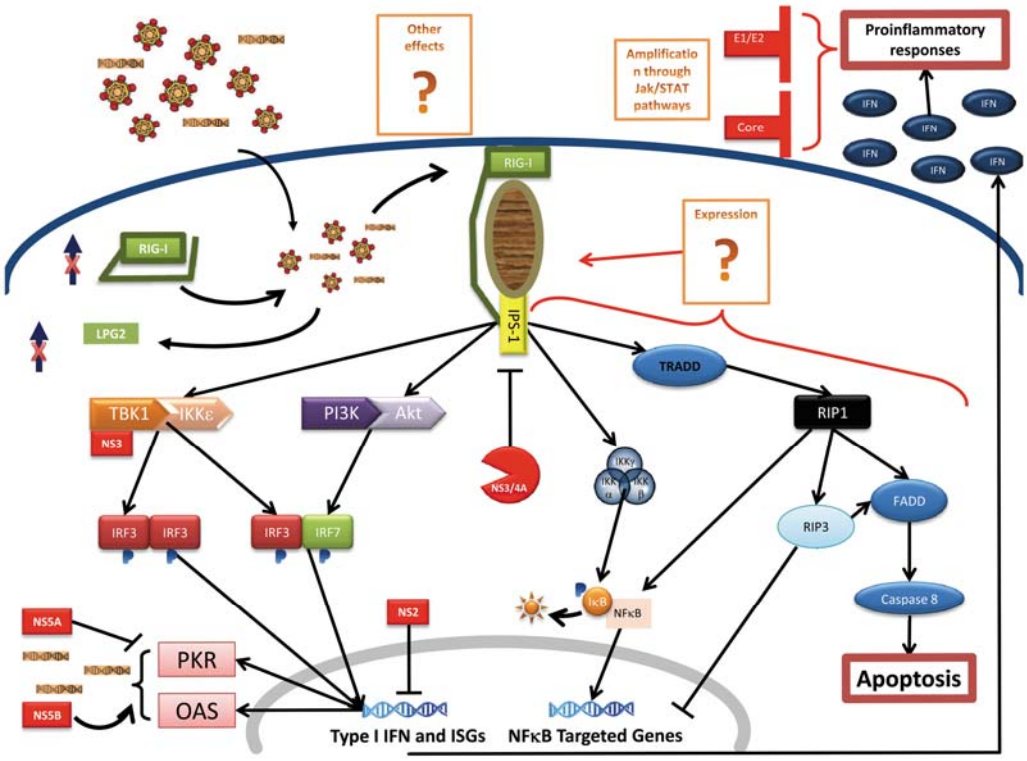

Figure 3. Schematic representation of HCV recognition and evasion by the RIG-I pathway. Viral entry and replication leads to the availability of genomic material in the cytosol of the infected cell that can be recognized by cytosolic receptors like RIG-I. Binding of RNA to it leads to a comformational change that exposes a CARD domain that helps it bind to IPS-1 (which needs to be mitochondria bound). Just like TRIF, IPS-1 is cleaved by NS3/4A which removes it out of the mitochondria and prevents downstream signaling. The components of the cascade below here are common with those of the TLR3 pathway. One notable observation is that, as with TLR3, the expression of RIG-I as well as other cytosolic receptors may be downregulated by the virus. LPG2, a molecule similar to RIG-I, has been said to be beneficial to the virus by competing with RIG-I although its expression has also been shown to be downregulated in chronic HCV biopsies.

to restore parts of the immune response to the virus and prevent its replication, indicating that IKKs can inhibit even in the absence of IFN. This may be because NS3/4A does not seem to proteolitically cleave neither TBK1 nor IKK epsilon, and even enhancement of their expression (by IFN alpha or TNF alpha addition to the media) fails to rescue the suppression in non-replicon cells (79, 89, 91). As for RIP-1, it seems its role in HCV is not thoroughly understood yet since it is related to viral induction of apoptosis, a new field in HCV research, and because it is not completely involved in the IFN/HCV interplay (75, 76, 91). Furthermore, it delays cell cycle progression and decreases cell growth rates out of which TLR4 seems to play a higher role.

4.4.2. Function of other TLR and PRRs in HCV

TLR3 is not the only receptor that recognizes HCV since cytosolic receptors RIG-I, PKR and 2^0-5^0 OAS have been shown to be involved in its recognition and are affected by the virus as well (80, 135, 136). Interestingly, these pathways have been shown not to be redundant and it is thought that perhaps crosstalk between these pathways may be the reason behind the varied modulatory effects observed after viral infection. This is highlighted by their varied expression across cells and tissues (79, 91, 106). HCV proteins themselves can also act as PAMP and induce IFN or modulate those responses (137). For instance, Core, NS3 and viral glycoproteins can interact and activate TLR2 or TLR4-mediated inflammation (117). The opposite has also been shown with NS5A were binding to the adaptor MyD88 and inhibit the recruitment of the kinase IRAK1 impairing the cytokine response (76, 117). It is important to fully understand not only how the viral RNA evades recognition but also how viral proteins can be recognized and may be engineered in the future to induce immunity against the virus during therapy.

4.5. Cytosolic receptors in the recognition of HCV

4.5.1. RIG-I and the cytosolic recognition of HCV RNA

There are other PRRs involved in the recognition of cytoplasmic viral RNA (particularly uncapped 5'-triphosphate motifs as well as RNA composition) that can lead to the induction of type I IFN (85, 105, 138-140). One of these is a member of a family of DexD/H box RNA helicases in the cytoplasm of cells named Retinoic Acid-Inducible Gene-I (RIG-I), which specifically binds to RNA secondary structures in the 5' or 3' NTR regions of HCV (Figure 3)(85, 106). Members of this family can be induced

by retinoic acid, IFNs and TNF alphą and are characterized by two-amino terminal caspase recruitment domains (CARD, critical for IFN induction) and a C-terminal helicase domain (91, 94). HCV is known to directly inhibit this pathway by the action of NS3/4A's protease activity on RIG-I (133, 134). The importance of this receptor in the recognition of HCV is highlighted by the cell line Huh7.5 which contains a mutation in the first CARD domain of RIG-I. While binding of dsRNA to the receptor still occurs, downstream signaling is abolished preventing the induction of IFN. Furthermore, while not yet addressed *in vitro*, RIG-I has been shown to be downregulated in biopsy samples of chronic HCV patients (89, 94). This is believed to be the reason why hepatocytes are permissive to this virus since the lack of RIG-I correlates well with increased viral replication plus a mutation in its CARD domain allows replicon HCV replication in a non permissive cell (106). Interestingly, current studies have failed to increase permissiveness in these cells merely by restoring RIG-I or Cardiff over-expression indicating that more is at play (79, 83, 85, 106). Furthermore, blocking IRF-3 did not increase replication efficiency of the virus in cell lines or in primary hepatocytes. The question remains as to the cause of such discrepancies: is it a technical problem such as a difference between stable transfection versus transient? Is it a reflection of the viral mechanism, such as the potency of the PAMP, or the effectiveness of the cleavage of IPS-1 by NS3/4A? Is it due to the presence or absence of other receptors or even a crosstalk between them not yet studied? One answer may be in the sequestration of dsRNA from RIG-I by its negative regulator LPG-2 but only in minor terms since this protein is also downregulated in response to HCV (85, 141, 142). Therefore, its real role in HCV remains unknown.

Just as with TLR3, the signaling cascade initiated by RIG-I leads to the activation of IRF3 and NF- kappa B leading to the induction of IFN beta (91). As a matter of fact, they both use the same signaling molecules although the exact reason behind their different behaviors is not fully understood. After dsRNA binding RIG-I undergoes a conformational change that uncovers the binding site for its CARD domain which is part of what drives the signal transduction (85, 106). This allows the association with itself and other molecules with the same domain on an adaptor protein that is mitochondria bound (85, 106, 107). This molecule known as either Cardif/IPS-1/MAVS/VISA (from here on we will refer to is as IPS-1 since it seems to be the most commonly used) undergoes its own conformational change exposing the binding sites for the IKKs which get recruited to the mitochondria (91, 94, 106, 107). Similarly to TRIF, the carbocy-terminal region of IPS-1 can activate the IKKα/β/γ complex, IKKε and TBK-1 (although IPS-1 seems to prefer the first one) and is broadly expressed in tissues (89, 91, 106). Out of these, the IFN pathway gets affected above IKK epsilon since its overexpression can inhibit HCV in replicon cells (94). Evidence for the activation of this pathway on HCV comes from studying stimulated (with LPS, PMA or dsRNA treatment) or infected cells transcriptome which reveals the upregulation of genes that are normally directly induced by IRF-3 such as RIG-I itself, ISG15, ISG1-8, ISG56, ISG54, CXCL10, Viperin, NOXA, RANTES CXCL11, and USP18 (75, 89). Other molecules, such as TRAF6, TRAF2, RIP1, FADD and TRAF3, can also interact with IPS-1 through a proline-rich region at its N-terminus (94). RIG-I is in turn regulated by this cascade, specifically IRAK1.

4.5.2. NS3/4A activity against RIG-I

Upon entry, the HCV polyprotein gives way to molecules necessary to perform duties for the propagation of the virus. Some of these proteins can have pathogenic effects on the cells apart from their normal replicative functions. In the case of HCV, non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B form a membrane bound complex for viral replication (86). While some of them are actually attached to the membranes, others are not or may localize to other places. NS3/4A, the protease/helicase complex, can localize also to the mitochondria where it serves a role in innate immune regulation (91, 107). NS2 can also inhibit the IFN promoter (although not as specifically as NS3/4A) eventually leading to the downregulation of different pro-inflammatory cytokines and chemokines like CCL5/RANTES and CXCL10/IP-10, but is not due to cleavage of IPS-1 (91). NS4B can instead induce the promoter activation although it has no viral functions currently ascribed to it and it does not co-localize with either the mitochondria or IPS-1 (112, 143). NS5A, a phosphoprotein, does not co-localize to the mitochondria with IPS-1 but with the ER membrane and inhibits the function of host antiviral proteins, for instance by binding to the kinase domain of PKR, by inhibiting IKK epsilon expression in the mitochondria (although no change in activity) or by modulating cell-cycle regulatory genes (89, 91, 99, 112). It can also serve after NF- kappa B activation by shifting to the induction of IL-8 which would down-regulate ISG expression. Core (which partially colocalizes with IPS-1) and other envelope proteins inhibit Jak-STAT pathway preventing the IFN amplification loops (99, 112, 144, 145). NS5B, the replicase, actually induces IFN probably by the production of dsRNA intermediates (even without the replication of the viral genome) but induces cell-cycle disruption by slowing the transition from S phase and the induction of IFN serves to make cells susceptible to DNA damage (75).

The protease NS3 can have more than one immunomodulatory function: apart from inhibiting IFN downstream of both TRIF and IPS-1, it is also able to bind TBK1 and act as a competitive inhibitor of this kinase, also evidenced by the fact that IKK epsilon overexpression can restore IFN activation (91, 99, 112). All of this leads to the conclusion that while most proteains have a role in immunomodulation, one of the most important viral factors in HCV is NS3/4A since it has the most impact on the innate immune response. Furthermore, there is a demonstrated interplay between RIG-I and HCV's NS3/4A protease where RIG-I have been demonstrated to provide important anti-viral immunity, while HCV has evolved ways to disrupt this response by interfering with both RIG-I and IPS-1 (116, 133, 134, 146). This is part of the reason why this particular interaction is studied since it can provide potential therapeutic targets against the virus (147). Furthermore, RIG-I may have an undetermined hepatic

function since knockout of this receptor *in vivo* leads to fetal death by massive liver degeneration (94). Viral RNA is recognized by RIG-I which induces IRF3 activation but becomes eventually overwhelmed after the protease levels manage to increase beyond a threshold (91, 107). It is suggested that RIG-I might recognize RNA duplexes which form later than viral proteins including NS3/4A, this would be in accordance to RIG-I being overwhelmed by the protease before IFN levels reach sufficient levels to clear the virus or induce an effective adaptive response (80, 83, 99, 104). Similar to other viral proteins, like 3ABC in HAV or NS3/4A in GBV-B, NS3/4A of HCV cleaves, in trans, within 5kDa of the short C-terminal transmembrane domain f IPS-1 at Cysteine 508 which dissociates it from the mitochondria and prevents downstream IRF-3 activation (80, 91, 99, 105-107, 112, 148). Consistent with this observation, the cleavage product of IPS-1 that moves from the mitochondria to the cytoplasm is also found on the cytoplasms of liver biopsies of chronic HCV patients (sera) and in the western blots of experimental cells (using genotype 1 or 2 HCV) (94, 102, 107). Furthermore, *in vitro* reproduction of these effects by siRNA silencing of IPS-1 mRNA not only prevents the IFN activation but leads to the enhancement of the HCV lifecycle in Huh7 cells and vice versa (83, 107). Conversely, other reports suggests that while RIG-I may play an important part in viral pathogenicity and development of chronicity, it might only be part of the story. First, the increase of permissiveness of Huh7 or Huh6 cells after addition of a dominant negative RIG-I was only marginal and did not reach the replication levels by Huh 7.5 cells (83). Second, while the role of TLR3 in this interplay has not been well established, it is clear that NS3/4A disrupts this pathway as well and furthermore there maybe communication between both pathways that may be disrupted by the viral protease, further increasing cellular susceptibility (75, 76, 91). Third, the fact that NS3/4A interferes with RIG-I pathway does not indicate this as the pathway for development of chronicity since the proteases of acute viruses like GBV-B and HAV have a similar function (102). Fourth, IFN alpha or TNF alpha treatment cannot enhance the expression of molecules in the TLR3 and RIG-I pathway after suppression with NS3/4A and increasing the expression of IPS-1 in cells can only partially overcome it (91).

4.5.3. PKR and 2'-5'OAS as separate receptors and as products of the IFN pathway in HCV

PKR and OAS are induced in response to the invading pathogens by the IFN pathway as ISGs (84, 149, 150). Conversely, their presence can increment the recognition of viral genomes and in so doing help amplify the response against it. Their role in HCV infection is not only denoted by their usual function but by the fact that NS5A and E1/E2 can directly bind either of these to prevent downstream signaling and amplification of proinflammatory responses (77, 86, 112, 151-153). In the case of PKR (a serine/threonine kinase) this binding prevents its autophosphorilation and dimerization inhibiting the activation of initiation factor 2a (eIF2a) which aids in protein translation, halts proliferation and induces apoptosis although these have not been found to be conducive to the suppression of HCV (76, 86, 154-159). OAS's function on the other hand is as a ribonuclease that destroys viral dsRNA and also it serves more as an effector against HCV by inducing RNAse L destruction of dsRNA although it is also believed to play a minor role (105, 160). Both of these receptors are constitutively expressed in an inactive form but their expression is upregulated by IFN. Conversely, a more prominent role for these cytosolic receptors should not be discarded as their role without NS3/4A evasion of TLR3 and RIG-I might be more prominent.

5. INNATE IMMUNE RECOGNITION AND THE INDUCTION OF CELL DEATH

The idea of direct cytopathogenicity caused by HCV has lately come to light even though the induction of inflammation caused by infection in the liver. The main reason for this lack of insight was due to the fact that histologic examination of biopsies did not show any apoptosis or necrosis and because the majority of chronic carriers are asymptomatic (161-164). It is now understood that virus immune evasion maybe one of the reasons that not overt cell death is observed since those samples are from patients were infection has formally established itself in the host and does not reflect an acute scenario (79, 99). In our case, the development of the cell line LH86 lead to the understanding that HCV may induce cell death in liver cells which does not happen in most other cells because they are selected for virus permissivity (87). Huh 7.5.1 cells were later also shown to have some level of apoptosis *in vitro* after virus infection although not extensive (69). The apoptotic bodies were capable of inducing a pro-inflammatory state by the induction of cytokines and the maturation of cocultured MoDCs. Even more interesting, is current non-HCV research that has developed the foundation to understand how the virus evades this branch of the innate response. It was discovered recently that death-domain containing RIP-1, which is normally associated with apoptosis by functioning downstream of TNFR by TRADD (a member of the TNF superfamily) interaction, can also associate with the TLR3 adaptor TRIF modulating the type of response (108, 117, 124, 125, 165). RIG-I also requires TRADD for downstream signaling and induces NOXA and ApoL6 (proapoptotic genes regulated by the IRF3, IKKs and IPS-1) indicating a role in the development of cell death of the infected cell (89, 102, 166). This would indicate that as long as both pathways are active (before evasion), type I IFNs, pro-inflammatory cytokines and apoptosis can come together to induce viral clearance. Once the virus interferes with these two pathways, the production or the synergy between these three effects disappears and may be a reason that cell death is not observed in chronic infection.

The question now remains, does the virus induce apoptosis by itself or is it a direct consequence of pathogen recognition? Certain groups have suggested the proapoptotic value of HCV proteins (like core or NS3) but it is debated whether this is a direct action or indirect due to the induction of IFN or pro-inflammatory cytokines (167-170). More importantly, some of these proteins can use apoptosis as a way to reduce the number of immune cells like DCs which has also been suggested to be detrimental

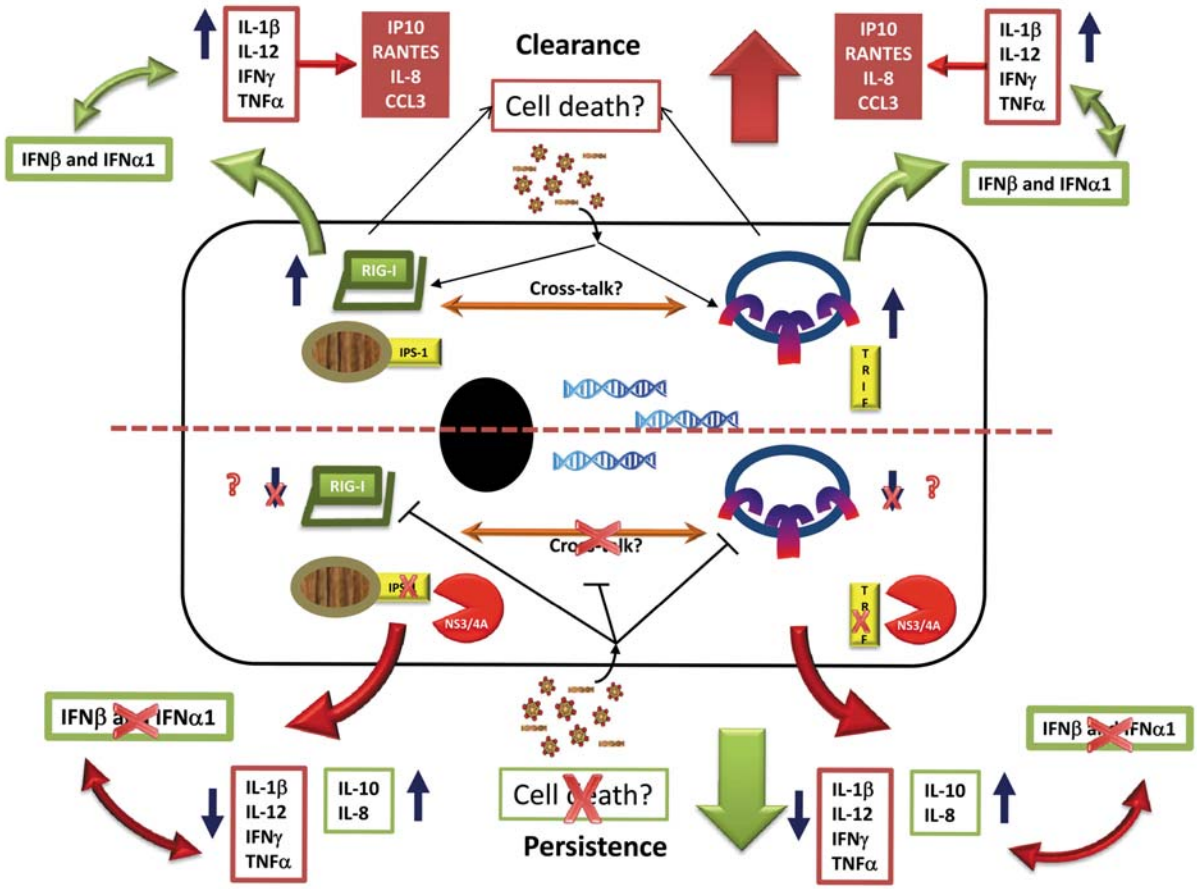

Figure 4. Model of acute interaction of hepatocytes with HCV. Based on data from the current literature, the interaction of HCV and the host innate response seems to be one of competition. Two scenarios are probably going on inside the cell at the same time. Upon virus binding and entry nucleic acid may be recognized by PRRs like TLR3 or RIG-I. The virus is going to induce the activation of the type I IFN cascade producing IFN beta and IFN alpha1 first. Throughout this process viral forces are attempting to prevent this activation and actively downregulating it either during binding with structural proteins or once inside through the production of NS3/4A cleaving both TRIF and IPS-1. Suggestion of crosstalk between these two pathways may indicate that the virus might also interfere with that processes to down modulate its own recognition. At some point the virus may also induce the downregulation of both RIG-I and TLR3 although when (or if it indeed happens should be investigated). If the virus is recognized and a strong IFN response is developed there would be in increase in type 1 pro-inflammatory cytokines and mobilizing chemokines which can lead to clearance but may induce cell death (although the smoking gun for this is still missing) and inflammation until then. Conversely, the virus could increase type 2 cytokines, like IL-10, tipping the balance and towards a more conducing environment for permanence.

to the hosts (171). Conversely, other groups have actually seen direct anti-apoptotic effects by proteins like NS2 and NS5A probably as an evasion mechanism by the virus (91, 172, 173). Perhaps cell death is not the cause of the inflammation as evidenced by the fact that TRADD-deficient mice can develop TNF induced hepatitis (166). One thing remains clear: there is a need to better understand the link between apoptosis and inflammation in order to gain a better understanding of how the virus balances recognition and evasion inside the host.

6. CONCLUDING REMARKS

It is increasingly clear that HCV is able to be recognized by the host cells and that furthermore the liver itself holds the key to its own cure. It is also clear that the infection has many faces instead of the "all-or-nothing" approach to how the virus interferes with the host. New research and data is leading the way to understanding a delicate balance between the host's immunity and the virus' arsenal (see Figure 4). A strong immunity, while it may be the cause of cell death and perhaps hepatitis, can be the key for viral clearance, while a weak response can be easily quenched by the virus to induce the chronic state. How to induce the first without incurring consequences for the host should be the focus of future research in the area of chronic hepatitis infection. Are there tools currently in use in other diseases that can be useful for shifting these balances? Do we need to come up with *de novo* solutions? Is it stopping one viral protein like NS3/4A the panacea, or does the

answer lie instead in one of the other proteins without our knowledge? Understanding the initial response to this virus is critical, and combined with preventative measures can help stave off chronicity. Induction of immunity by transduced DCs, increasing PRR expression or activating alternative pathways can lead to an early disruption of the viral life cycle and promote clearance. Perhaps the answer is already here and all we need to do is simply understand it.

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