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Review

Lymphocyte Distribution and Intrahepatic Compartmentalization during HCV Infection: a Main Role for MHC-Unrestricted T Cells

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Abstract. Hepatitis C virus (HCV) infection induces an acute and chronic liver inflammation through an immune--mediated pathway that may lead to cirrhosis and liver failure. Indeed, HCV-related hepatitis is characterized by a dramatic lymphocyte infiltrate into the liver which is mainly composed by HCV non-specific cells. Several data indicated that interferon (IFN)-γ secretion by intrahepatic lymphocytes (IHL) may drive non-specific cell homing to the liver, inducing interferon inducible protein-10 (IP-10) production. An interesting hallmark of these IHL is the recruitment of lymphocytes associated with mechanisms of innate immunity, such as natural killer (NK), natural killer T (NKT) and γδ T lymphocytes. CD81 triggering on NK cell surface by the HCV envelope glycoprotein E2 was recently shown to inhibit NK cell function in the liver of HCV-infected persons, resulting in a possible mechanism contributing to the lack of virus clearance and to the establishment of chronic infection. In contrast, intrahepatic NKT cells restricted to CD1d molecules expressed on the hepatocyte surface may contribute to a large extent to liver damage. Finally, an increased frequency of T cells expressing the γδ T cell receptor (TCR) was observed in HCV-infected liver and recent observations indicate that intrahepatic γδ T cell activation could be directly induced by the HCV/E2 particle through CD81 triggering. These cells are not HCV specific, are able to kill target cells including primary hepatocytes and their ability to produce T helper (Th)1 cytokines is associated with a higher degree of liver disease. Together, CD1d/NKT and/or E2/CD81 interactions may play a major role in the establishment of HCV immunopathogenesis. In the absence of virus clearance, the chemokine-driven recruitment of lymphocytes with an innate cytotoxic behavior in the liver of HCV-infected patients may boost itself, leading to necroinflammatory and fibrotic liver disease.

Key words: hepatitis C; liver; intrahepatic lymphocytes; NK cells; NKT cells; NT cells; γδ T lymphocytes; HLA; CD1; CD81; E2.

Abbreviations used: HBV – hepatitis B virus, HCV – hepatitis C virus, HIV – human immunodeficiency virus, EBV – Epstein-Barr virus, NK – natural killer, IP-10 – interferon inducible protein-10, NKT – natural killer T, NT – natural T, MHC – major histocompatibility complex, ConA – concanavalin A, α-GalCer – α-galactosylceramide.

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The hepatitis C virus (HCV) is a small, positive--stranded RNA virus, identified as the leading causative agent of post-transfusional and community-acquired non-A, non-B viral hepatitis[23]. The majority of HCV infections result in chronic hepatitis, often progressing to cirrhosis and/or hepatocellular carcinoma. The high percentage of chronicity may be due to active escape mechanisms of the virus[50, 100] or to HCV-induced altered immune responses. In contrast, less than 10% of individuals infected with hepatitis B virus will develop the chronic disease[22]. This difference may imply a diversity in the "survival strategies" of hepatitis viruses and/or distinctions in host immune responses against HCV versus HBV. The E2-HCV protein seems to be a central component of distinct HCV evasion mechanisms[34, 86]. There is no vaccine for HCV infection and the only available therapy, interferon (IFN)-α and ribavirin, has proven efficacious in less than 50% of patients[58].

HCV infection is characterized by a dramatic lymphocyte infiltration into the liver[7, 62]. However, the majority of liver-infiltrating lymphocytes are not HCVspecific. At present, the role played by lymphocytes recruited to the liver in terms of protection and/or pathogenesis of HCV-induced hepatitis is not well understood. Several lines of evidence argue in favor of immune-mediated hepatic damage triggered by infiltrating lymphocytes rather than a direct HCV-mediated cytopathic effect[6, 64, 101]. In the acute phase intrahepatic lymphocytes (IHL) could be critical for the resolution of the disease, whereas in the chronic phase IHL could be dangerous, damaging infected or uninfected hepatocytes through both the direct killing of liver cells and the release of T helper (Th)1 cytokines. The production of IFN-γ at the site of infection may have two opposite effects: an important antiviral activity or an efficient cytotoxic activity against hepatocytes[40]. In this respect, animal models of experimentally induced hepatitis support the hypothesis that the secretion of Th1 cytokines is a critical mechanism in inducing hepatic injury[6, 61]. For example, in a model of transgenic mice expressing IFN-γ under the control of a liver-specific promoter, the animals developed chronic hepatitis[90]. Furthermore, IFN-γ has been shown to be the principal mediator of the hepatic inflammatory process induced against hepatitis B surface antigen expressed in the liver of transgenic mice[3]. Finally, the expression of interleukin 2 (IL-2) and IFN-γ in the liver of HCV^+ persons positively correlated with the extent of hepatic fibrosis and portal inflammation[64].

Cell recruitment from the blood stream requires the concerted action of several adhesion molecules: integrins, immunoglobulin-like molecules, selectins, and glycoproteins serving as selectin ligands. Selectin-mediated adhesion represents the first step in the cascade required for leukocyte recruitment. Specifically, the L-selectin (CD62L) has been shown to be involved in leukocyte rolling, which is a transient adhesion event during early inflammation that allows the lymphocytes to migrate to the inflamed tissue. Two compartments could be discriminated at the single cell level on the basis of the expression of adhesion molecules: peripheral blood lymphocytes (PBL) and IHL (Fig. 1). Naive cells expressing the CD62L are predominant in the peripheral blood. In contrast, IHL do not express this adhesion molecule necessary for extravasation and migration into the inflamed tissue. Intrahepatic cells show an increased expression of class II MHC molecules compared with PBL, indicating an activated phenotype[68]. The analysis of naive, central and effector memory T cell subsets in the HCV^+ liver showed an enrichment of $CCR7^-$ cells, suggesting a liver compartmentalization of activated/effector cells. Using these criteria for discriminating IHL from PBL contamination, an enrichment of lymphocytes with a cytotoxic behavior was observed in the liver of HCV-infected persons[1, 2, 66]. Specifically, an interesting hallmark of the liver of patients experiencing HCV-related hepatitis is the recruitment of lymphocytes associated with mechanisms of innate immunity[66]. The innate immune system uses only a small, relatively inflexible, cell population composed of natural killer (NK) cells, natural killer T (NKT) cells and γδ T cells. This innate activity of the immune system provides early antimicrobial immunity and is able to determine the nature of the downstream adaptive immune response.

In the course of HCV infection, the frequency of intrahepatic NK cells, NKT cells, and T cell receptor (TCR) γδ T cells (Vδ1 and Vδ2) was significantly higher than in the peripheral blood of the same patients (Fig. 2). In contrast to classical MHC-restricted recognition of antigens, NK lymphocytes can "see" and kill target cells deficient in the expression of one or more MHC class I molecules. This cell subset provides an important defense line against viruses through a rapid and potent cytotoxic activity and the release of antiviral cytokines. Moreover, the IFN-γ released by NK cells is able to directly inhibit HBV replication and drive the Th cell response to a Th1 profile[40]. NK function is negatively and positively regulated through a variety of receptors, most of which are known to interact with MHC class I molecules[12, 24, 55, 63, 77]. A dynamic and coordinated balance between activating and inhibitory receptors controls NK cell functions and influences the

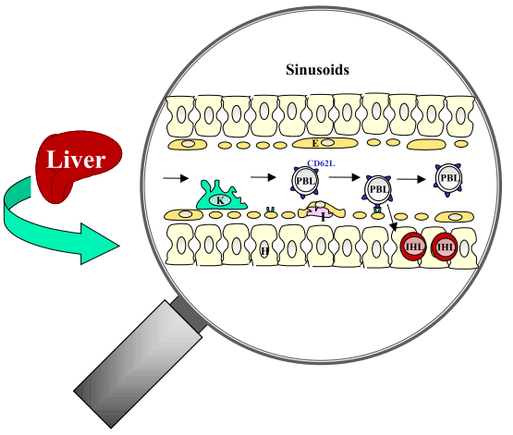

Fig. 1. Schematic representation of liver section. Intrahepatic lymphocytes (IHL) circulating in liver sinusoid and/or migrating in liver tissue. H – hepatocytes, I – Ito cells, K – Kuppfer cells, ➧ – CD62L

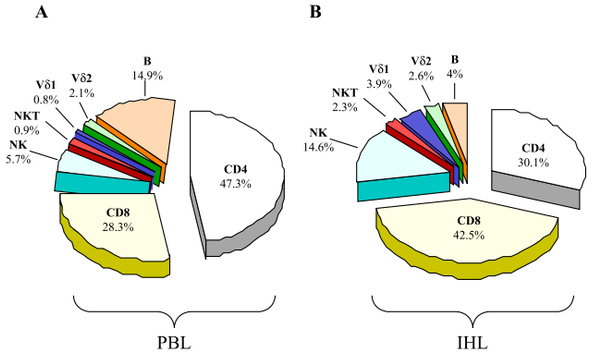

Fig. 2. Lymphocyte distribution in the peripheral blood and in the liver of HCV-infected patients. Panel **A** – PBL. Panel **B** – IHL

selective recognition of virus-infected, tumor or allogeneic cells. In HCV infection, it was shown that both the virus and the chronic inflammation modulate NK receptor expression on the liver-infiltrating lymphocytes, regulating their immune response to the infection[97]. NK cells can also modulate the adaptive response by releasing IFN-γ and a wide variety of cytokines and immunoregulatory mediators[78].

In humans, an impairment of NK functions has been associated with an increased susceptibility to different viral infections, such as herpes simplex virus, Epstein-Barr virus (EBV), cytomegalovirus and human immunodeficiency virus (HIV)[2, 18, 59]. Similar results were shown also in chronic HCV patients, confirming a critical role of NK-mediated immunity versus viral infections[25]. Accordingly, in HBV infection, GUIDOTTI et al.[41], showed that early IFN-γ production by NK cells at the site of infection is a critical event in response to the virus. In particular, in acutely HBV-infected chimpanzees, NK cells were able to control HBV replication before the peak of T cell infiltration. Thus, NK activity rather than CD3-mediated immunity was an early critical component in the protection from HBV infection. In contrast, in chronic HCV infection, the NK cell function was shown to be significantly decreased compared with healthy donors[25]. The mechanism responsible for this HCV-induced NK cell impairment is not well understood. Recently, TSENG et al.[92] proposed CD81 triggering as a possible candidate to inhibit NK cell functions through a novel negative signaling mechanism[26]. HCV interacts with NK cells via E2-CD81 interactions, resulting in a direct inhibition of NK cell function. Specifically, *in vitro* ligation of CD81 on NK cells by anti-CD81 or by immobilized E2 (anti-E2-rHCV-E2) blocks NK activation, inhibits cytokine production after exposure to different cytokines (IL-2, IL-12, IL-15) or by CD16 crosslinking. Moreover, CD81 crosslinking is able to block the cytotoxic granule release induced by CD16 and to reduce IL-2 induced proliferation, suggesting that HCV could interact with NK cells through E2-CD81 interaction. This mechanism may influence the innate immune response to HCV infection and, therefore, the kinetics and magnitude of T and B cell immunity. Accordingly, an imbalance of Th1 versus Th2 responses has been proposed as a possible mechanism responsible for the viral persistence seen in chronic HCV infection.

Along with classical NK cells, the HCV-infected liver shows an enrichment of cells expressing both NK-like and T cell-like recognition structures. These T cell subsets have a highly restricted TCR repertoire (both αβ and γδ), are frequently double negative (DN) for CD4 and CD8 and may recognize glycolipid antigens in the context of the CD1 molecule. Human CD1 proteins are HLA class I-like molecules that can be divided into two separate groups: group 1 comprising CD1a, CD1b and CD1c and group 2 containing the more divergent CD1d[16, 75]. Group 1 CD1 proteins are mainly expressed on many specialized antigen-presenting cells (APC), including Langerhans cells in the epidermis and dendritic cells in different organs[37, 60, 65], whereas group 2 CD1 molecules are expressed on the gastrointestinal epithelium and hematopoietic cells[9, 10]. Immunohistochemical analysis confirmed that HLA class I is poorly expressed on hepatocytes (data not shown), suggesting that CD8 T cell recognition of viral peptides in the context of HLA class I molecules may not represent the main cytolytic pathway occurring in the liver. Accordingly, immunohistochemical analysis of CD1a, -b, -c and -d expression in the liver showed that hepatocytes express high levels of CD1d molecule independently of HCV infection, suggesting that CD1d could be the main

restriction element in the liver (AGRATI et al., manuscript in preparation).

αβ NKT cells express a TCR composed of a single invariant TCR α-chain (Vα14, Jα281 in mice and Vα24, JαQ in humans) and a highly skewed TCR β-chain (Vβ11 in the humans and Vβ8 in the mouse)[4, 29, 33, 53]. NKT cell activation results in a rapid production of cytokines (IL-4 and IFN-γ), cell proliferation and NK-like cytotoxicity[5, 42, 48]. Moreover, it was reported that stimulation of NKT cells is able to rapidly induce activation of innate (NK cells) and adaptive (T cells and B cells) immune responses[18]. This αβ T cell subset expresses NK-like markers belonging to the NKRP1 family and recognizes glycolipid antigens presented by CD1d molecules[76].

In the liver of HCV-infected patients an increase of Vα24 NKT cells (up to 20-fold in comparison with the peripheral blood) was observed[66]. The memory/activated phenotype associated with the oligoclonal expansion suggests that these NKT cells may recognize an endogenous ligand or ubiquitous antigen. The endogenous ligand could normally be expressed in the liver of healthy persons, or processed and presented in the context of CD1d to Vα24/Vβ11 TCR-expressing cells as a consequence of tissue damage. This subset is able to produce high amounts of Th1 cytokines rapidly and to kill other cells with an NK-like mechanism. Recent findings have suggested a pathological role for NKT cells based on their ability to produce large amounts of IFN-γ. It has been reported that Vα14 NKT cells play a critical role in various diseases, including *Salmonella* infections[45], autoimmune diabetes[102] and systemic sclerosis[81]. In a murine model of hepatitis induced by intravenous injection of concanavalin A (ConA), the liver injury is strictly associated with the presence of lymphocyte infiltrates. The absence of liver injury in athymic nude mice or SCID mice indicates the immunopathological origin of ConA-induced hepatitis[87]. Several observations demonstrate that only Vα14$^+$ NT cells are required for the development of ConA-induced hepatitis[46]. For example, the antibody depletion of NK1.1$^+$ cells *in vivo* confers resistance to hepatitis, and this resistance is also present in β2 microglobulin or CD1 knock-out mice that have dysfunctional NK1.1$^+$ NKT cells[83, 89]. Moreover, it was demonstrated that NKT cells critically contribute to liver damage caused by the generalized Shwartzman reaction[67], suggesting a general involvement of NKT cells in immune-mediated hepatitis. Although the contribution of NKT cells to HCV-induced hepatitis remains to be determined in further studies, we speculate that they are involved in mediating the liver injury observed in HCV$^+$ persons, not only by itself[83], but also by cooperating with conventional T cells[49, 95] and macrophages[87]. Vα14 T cells seems to explain their effector activity through several mechanisms, such as Fas-FasL interactions[82], the perforin-granzyme system[99] and IFN-γ release[51, 82].

In the HCV$^+$ liver, an increased fraction of T cells expressing γδ TCR was also observed[1, 66, 97]. γδ T cells represent a minor population of human peripheral lymphocytes (3–6%)[27] and express the CD16 NK marker, indicating their natural T (NT) nature[71]. The majority of these cells express the Vδ2 TCR variable segment associated with the Vγ9 segment[69]. Vγ9Vδ2 NT lymphocytes recognize phosphorylated nonpeptidic microbial metabolites[14, 15, 85] and alkylamines[71] without the requirement of antigen uptake or processing, or MHC class I or class II expression[84]. Also, γδ NT cells have been shown to release high levels of cytokines such as IFN-γ, TNF-α[52], and β chemokines involved in the recruitment of cells of the monocyte/macrophage lineage during an inflammatory reaction. NK receptors expressed on Vγ9Vδ2 NT cell surfaces sharply regulate their activity[38, 73, 74] and influence γδ responses in antiviral reactivity, tumor immunity and autoimunity[36, 73]. In contrast, Vδ1 NT cells represent a minor lymphocyte subpopulation in the peripheral blood, usually expressing a naive phenotype in healthy donors. Their Vδ1-encoded receptor chain is typically co-expressed with Vγ--encoded chains distinct from Vγ9[91]. However, Vδ1$^+$ cells are the predominant γδ T cell population in the postnatal thymus[20] and represent a major T cell population in the skin, intestinal and pulmonary epithelium. Unlike αβ T cells, which re-circulate extensively, γδ T cells in these epithelial tissues seem to remain immobile. The selective expression of TCR V-gene segments in different epithelial tissues is also observed in mice[32, 72] and may reflect the possibility that these T cells are specialized in responding to certain types of antigens expressed at these sites. Specifically, Vδ1 T cells represent the major T cell subpopulation in the human intestine[28, 30, 70, 96], suggesting their possible role as the first line of defense against the invading microbes entering the gastrointestinal tract.

The ligands recognized by Vδ1 NT lymphocytes are stress antigens of cellular origin. Vδ1$^+$ NT cells recognize and interact with the MICA and the closely related MICB glycoproteins that are expressed mainly in the gastrointestinal and thymic cortical epithelium and on hepatocyte-derived cell lines. The MIC genes have heat-shock response elements in their promoters and show a low homology with MHC class I molecules[39]. The stress-induced expression of MICA and MICB, and their recognition by polyclonal Vδ1 NT cells

through TCR or the NKG2-D natural killer receptor, may serve as an immune surveillance mechanism for detecting damaged, infected, or transformed intestinal epithelial cells. Moreover, tissue Vδ1 NT lymphocytes were recently shown to recognize nonpolymorphic CD1c molecules[80]. Specifically, Vδ1 NT cells were found to proliferate and release Th1 cytokines in response to $CD1^+$-presenting cells and to lyse $CD1c^+$ targets. The recognition of CD1c is TCR mediated and dependent on Vδ1 TCR expression. The reactivity of γδ NT cell lines and clones to CD1c is highly specific and independent of the presence of exogenous antigen. In the course of HCV infection, γδ T cells are recruited to the liver[1, 66, 97]. In particular, phenotypic analysis of this T cell subset indicated that this increase is due to $CD3^+$ cells expressing the Vδ1 chain of the TCR and results in an inversion of the intrahepatic Vδ2 to Vδ1 ratio[1]. Interestingly, Vδ1 T cells homing to the liver were specifically induced by HCV infection: indeed, the analysis of HCV^- persons failed to show any Vδ1 compartmentalization in the liver[2]. The rapid recruitment of Vδ1 to the liver of HCV-infected persons may be driven by the recognition of HCV antigens and/or by alterations of the cytokine and chemokine environment. Indeed, in HIV infection, the increase of the Vδ1 T cell subset observed in the blood suggests that HIV infection induces a Vδ1 mobilization from the mucosa tissue to the periphery under the influence of various cytokines and/or chemokines. Accordingly, as a result of viral interference, in HIV/HCV co-infected persons this increased fraction of peripheral Vδ1 T cells tends to home to the site of HCV infection, confirming a recruitment to the liver driven by HCV[2].

The exact role played by γδ T cells in the protection and/or pathogenesis in HCV infection is not well understood. However, the involvement of γδ T lymphocytes in immunosurveillance has been suggested in several infections with herpesviruses, including herpes simplex virus[13] and cytomegalovirus[27]. An increased number of γδ T cells has also been observed in animal models of influenza[17] and Sendai virus infection[44] as well as in patients with EBV and HIV[57, 98]. Several reports have provided strong evidence of the anti-inflammatory role of γδ T cells through the homeostatic regulation of αβ T cells[32]. Thus, the rapid homing of circulating γδ NT cells to the liver may determine the pattern of the adaptive responses mediated by the subsequent activation of MHC-restricted αβ T lymphocytes. Interestingly, the Vδ1 T cells recruited to the liver display an activated/memory phenotype ($CD62L^-CD45RO^+CD95^+$) suggesting an antigen-mediated stimulation. However, the lack of selective expression of any Vγ-chain indicates a polyclonal "superantigen-like" activation. Intrahepatic γδ T lymphocytes from HCV^+ persons can be expanded *in vitro* with a cytokine cocktail containing IL-2, IL-4, IL-7 and IL-15[93]. γδ T cell lines obtained from HCV-infected livers show a potent MHC-unrestricted cytotoxic activity against NK-sensitive (K562), NK cell-resistant (Daudi and Huh7) targets and, finally, against primary hepatocytes. In contrast, αβ T cell lines from the liver of the same patients failed to kill any of the target cells tested[93]. Nevertheless, intrahepatic γδ T cells recruited to the liver under the influence of HCV infection were not specific for HCV antigens. Finally, none of the intrahepatic γδ T cell lines were able to kill autologous EBV-transformed B cells infected with recombinant vaccinia viruses expressing HCV proteins, indicating that this T cell subset does not recognize HCV proteins. Proliferative experiments demonstrated that γδ T cells were unable to respond to structural (Core, E1, E2) and nonstructural (NS3, NS4, NS5) HCV recombinant proteins, confirming that intrahepatic γδ T cells are not specific for HCV proteins or HCV-infected cells[93]. Thus, intrahepatic Vδ1 T lymphocytes can contribute to liver pathology by killing hepatocytes without HCV specificity. An analogous MHC-unrestricted and viral non-specific cytotoxic activity exerted by γδ T lymphocytes on cells infected with HIV and herpes simplex virus has been reported[13, 47, 56].

Another important event in the induction of necroinflammatory processes in the liver is the release of inflammatory cytokines[68]. In HCV infection, *ex vivo* stimulation of intrahepatic lymphocytes showed an increased frequency of IFN-γ producing Vδ1 T cells compared with peripheral Vδ1 T lymphocytes from the same donors. Cytokine analysis of γδ T cell lines obtained from HCV^+ livers confirmed the ability of this T cell subset to release high levels of Th1 cytokines[93]. Interestingly, the percentage of IFN-γ-releasing Vδ1 T cells is higher in the liver of HCV^+ persons going through the necroinflammatory process, suggesting that the overall role of Vδ1 T lymphocytes in the HCV^+ liver produces pathogenic rather than protective results[1]. Thus, Vδ1 T lymphocytes homing to the liver of HCV^+ persons may contribute to the immunopathogenesis of HCV-induced hepatitis through two distinct mechanisms: killing infected and uninfected hepatocytes without HCV specificity and releasing inflammatory cytokines. The possible ligand of Vδ1 T cells are heat shock proteins or stress proteins of cellular origin[34, 39]. Possible candidates are MHC-related proteins MICA and MICB that could function as self antigens and are recognized broadly by intestinal epithelial

Vδ1 T cells through TCR or the NKG2-D natural killer receptor without Vγ restriction. The recognition of stress-induced proteins on the surface of hepatocytes could be an important mechanism in the immune surveillance mechanism for the detection of HCV-infected cells. The CD1c was shown to be another possible ligand, but it is expressed on the hepatocyte' surface neither in the uninfected nor in the HCV^+ liver. More recently, TSENG et al.[93], showed that *in vitro* stimulation of intrahepatic γδ T cell lines by immobilized anti-CD81 induced a release of significant levels of TNF-α and IFN-γ indicating that CD81 may be a crucial molecule in γδ T cell activation in HCV infection.

CD81 is a member of the tetraspan superfamily of proteins. CD81 is a 26 kDa protein composed of 4 transmembrane and 2 extracellular domains. It is expressed on most human tissues, and within a single tissue its levels vary during development and in response to cellular activation[54]. A common characteristic of tetraspanins, including CD81, is a propensity to associate physically with a variety of other membrane proteins to form signal transduction complexes. Ligation of CD81 with monoclonal antibodies results in a costimulatory signal for B and T cells expressing αβ TCR. CD81 molecule associates CD19, CD21 and Leu-13[11, 35, 43] on the B cell surface to form a multimolecular complex that reduces the threshold for B cell activation[19, 35]. On the T lymphocyte surface, CD81 is expressed in association with the CD4 and CD8 molecules[88] and acts as a costimulatory signal for proliferation and cytokine production[79, 103]. γδ T cells, which lack CD4 and CD8 coreceptors, also express CD81 on their surface but respond differently to CD81 crosslinking than do αβ T cells. Indeed, crosslinking of CD81 on γδ T lymphocytes results in direct cell activation without the need of CD3 stimulation[94]. Moreover, recent data demonstrated a third different pathway of CD81-mediated signaling, inducing inhibition of NK functions (cytokine production, cytotoxicity and proliferation).

Altogether, several data suggest that innate (non-specific) immunity plays an important role in the liver pathology of HCV infection. Intrahepatic production of Th1 cytokines and chemokines by HCV infection promotes the recruitment of non-specific lymhocytes. These innate cells are directly recruited to the infected liver by the inflammatory process, without the need for antigen recognition and clonal expansion in the regional lymph nodes. This process requires the concerted action of several adhesion molecules and results in the enrichment of lymphocytes with innate cytotoxic behavior. In the absence of viral clearance, this pathway will boost itself, leading to necroinflammatory and fibrotic liver disease. The molecular mechanisms may involve CD1d/NKT and/or E2/CD81 interactions, promoting the intrahepatic recruitment of non-specific T cells releasing proinflammatory cytokines. Thus, new strategies for therapeutic intervention could be directed to inhibit the molecular interactions responsible for the recruitment and non-specific activation of IHL in HCV-infection.

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